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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A23L 1/0524, A23C 9/137, 9/154, 21/08	A1	(11) International Publication Number: WO 97/03574 (43) International Publication Date: 6 February 1997 (06.02.97)
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(54) Title: PROCESS FOR STABILIZING PROTEINS IN AN ACIDIC ENVIRONMENT WITH A HIGH-ESTER PECTIN		
(57) Abstract A process is described wherein there is added to an acidic environment, which contains at least one protein, a block-wise enzymatically de-esterified pectin, and wherein the pectin is a high ester pectin. Also described is a recombinant pectin methyl esterase.		

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PROCESS FOR STABILIZING PROTEINS IN AN ACIDIC ENVIRONMENT WITH A
PECTIN HIGH-ESTER

The present invention relates to a process and an enzyme for use in such a process.

5 In particular, the present invention relates to a process for preparing and using an enzymatically modified pectin.

Pectin is an important commodity in today's industry. For example, it can be used in the food industry as a thickening or gelling agent, such as in the preparation of
10 jams.

Pectin is a structural polysaccharide commonly found in the form of protopectin in plant cell walls. The backbone of pectin comprises α -1-4 linked galacturonic acid residues which are interrupted with a small number of 1,2 linked α -L-rhamnose units.
15 In addition, pectin comprises highly branched regions with an almost alternating rhamno-galacturonan chain. These highly branched regions also contain other sugar units (such as D-galactose, L-arabinose and xylose) attached by glycosidic linkages to the C3 or C4 atoms of the rhamnose units or the C2 or C3 atoms of the galacturonic acid units. The long chains of α -1-4 linked galacturonic acid residues
20 are commonly referred to as "smooth" regions, whereas the highly branched regions are commonly referred to as the "hairy regions".

Some of the carboxyl groups of the galacturonic residues are esterified (e.g. the carboxyl groups are methylated). Typically esterification of the carboxyl groups
25 occurs after polymerisation of the galacturonic acid residues. However, it is extremely rare for all of the carboxyl groups to be esterified (e.g. methylated). Usually, the degree of esterification will vary from 0-90%. If 50% or more of the carboxyl groups are esterified then the resultant pectin is referred to as a "high ester pectin" ("HE pectin" for short) or a "high methoxyl pectin". If less than 50% of the
30 carboxyl groups are esterified then the resultant pectin is referred to as a "low ester pectin" ("LE pectin" for short) or a "low methoxyl pectin". If the pectin does not contain any - or only a few - esterified groups it is usually referred to as pectic acid.

The structure of the pectin, in particular the degree of esterification (e.g. methylation), dictates many of the resultant physical and/or chemical properties of the pectin. For example, pectin gelation depends on the chemical nature of the pectin, especially the degree of esterification. In addition, however, pectin gelation also depends on the soluble-solids content, the pH and calcium ion concentration. With respect to the latter, it is believed that the calcium ions form complexes with free carboxyl groups, particularly those on a LE pectin.

Pectic enzymes are classified according to their mode of attack on the galacturonan part of the pectin molecule. A review of some pectic enzymes has been prepared by Pilnik and Voragen (Food Enzymology, Ed.: P.F.Fox; Elsevier; (1991); pp: 303-337). In particular, pectin methylesterases (EC 3.1.1.11), otherwise referred to as PMEs, de-esterify HE pectins to LE pectins or pectic acids. In contrast, and by way of example, pectin depolymerases split the glycosidic linkages between galacturonosyl methylester residues.

In more detail, PME activity produces free carboxyl groups and free methanol. The increase in free carboxyl groups can be easily monitored by automatic titration. In this regard, earlier studies have shown that some PMEs de-esterify pectins in a random manner, in the sense that they de-esterify any of the esterified (e.g. methylated) galacturonic acid residues on more than one of the pectin chains. Examples of PMEs that randomly de-esterify pectins may be obtained from fungal sources such as *Aspergillus aculeatus* (see WO 94/25575) and *Aspergillus japonicus* (Ishii *et al* J Food Sci 44 pp 611-14). Baron *et al* (Lebensm. Wiss. M-Technol 13 pp 330-333) apparently have isolated a fungal PME from *Aspergillus niger*. This fungal PME is reported to have a molecular weight of 39000 D, an isoelectric point of 3.9, an optimum pH of 4.5 and a K_m value (mg/ml) of 3.

In contrast, some PMEs are known to de-esterify pectins in a block-wise manner, in the sense that it is believed they attack pectins either at non-reducing ends or next to free carboxyl groups and then proceed along the pectin molecules by a single-chain mechanism, thereby creating blocks of unesterified galacturonic acid units which are

very calcium sensitive. Examples of such enzymes that block-wise enzymatically de-esterify pectin are plant PME's. Up to 12 isoforms of PME have been suggested to exist in citrus (Pilnik W. and Voragen A.G.J. (Food Enzymology (Ed.: P.F.Fox); Elsevier; (1991); pp: 303-337). These isoforms have different properties.

5

Versteeg *et al* (J Food Sci 45 pp 969-971) apparently have isolated a PME from orange. This plant PME is reported to occur in multiple isoforms of differing properties. Isoform I has a molecular weight of 36000 D, an isoelectric point of 10.0, an optimum pH of 7.6 and a K_m value (mg/ml) of 0.083. Isoform II has a
10 molecular weight of 36200 D, an isoelectric point of 11.0, an optimum pH of 8.8 and a K_m value (mg/ml) of 0.0046. Isoform III (HMW-PE) has a molecular weight of 54000 D, an isoelectric point of 10.2, an optimum pH of 8 and a K_m value (mg/ml) of 0.041. However, to date there has been very limited sequence data for such PME's.

15

According to Pilnik and Voragen (*ibid*), PME's may be found in a number of other higher plants, such as apple, apricot, avocado, banana, berries, lime, grapefruit, mandarin, cherries, currants, grapes, mango, papaya, passion fruit, peach, pear, plums, beans, carrots, cauliflower, cucumber, leek, onions, pea, potato, radish and
20 tomato. However, likewise, to date there has been very limited sequence data for such PME's.

25

Random or blockwise distribution of free carboxyl groups can be distinguished by high performance ion exchange chromatography (Schols *et al* Food Hydrocolloids 1989 6 pp 115-121). These tests are often used to check for undesirable, residual
PME activity in citrus juices after pasteurisation because residual PME can cause, what is called, "cloud loss" in orange juice in addition to a build up of methanol in the juice.

30

PME's have important uses in industry. For example, they can be used in or as sequestering agents for calcium ions. In this regard, and according to Pilnik and Voragen (*ibid*), cattle feed can be prepared by adding a slurry of calcium hydroxide

to citrus peels after juice extraction. After the addition, the high pH and the calcium ions activate any native PME in the peel causing rapid de-esterification of the pectin and calcium pectate coagulation occurs. Bound liquid phase is released and is easily pressed out so that only a fraction of the original water content needs to be removed by expensive thermal drying. The press liquor is then used as animal feed.

Pilnik and Voragen (*ibid*) list uses of endogenous PMEs which include their addition to fruit juices to reduce the viscosity of the juice if it contains too much pectin derived from the fruit, their addition as pectinase solutions to the gas bubbles in the albedo of citrus fruit that has been heated to a core temperature of 20 to 40°C in order to facilitate removal of peel and other membrane from intact juice segments (US-A-4284651), and their use in protecting and improving the texture and firmness of several processed fruits and vegetables such as apple (Wiley & Lee 1970 Food Technol 24 1168-70), canned tomatoes (Hsu *et al* 1965 J Food Sci 30 pp 583-588) and potatoes (Bartolome & Hoff 1972 J Agric Food Chem 20 pp 262-266).

Glahn and Rolin (1994 Food Ingredients Europe, Conf Proceedings pp 252-256) report on the hypothetical application of the industrial "GENU pectin type YM-100" for interacting with sour milk beverages. No details are presented at all on how GENU pectin type YM-100 is prepared.

EP-A-0664300 discloses a chemical fractionation method for preparing calcium sensitive pectin. This calcium sensitive pectin is said to be advantageous for the food industry.

Thus, pectins and de-esterified pectins, in addition to PMEs, have an industrial importance. However, there is a continuing need to improve the known methods of stabilising proteins in an acidic environment but without adversely affecting the viscosity of that environment. In this regard, an adverse effect on the viscosity of the environment can compromise the overall appearance and/or texture and/or palatability and/or mouth feel of the resultant product.

According to a first aspect of the present invention there is provided a process comprising adding to an acidic environment, which contains at least one protein, a block-wise enzymatically de-esterified pectin, wherein the pectin is a high ester pectin.

5

According to a second aspect of the present invention there is provided a method of block-wise enzymatically de-esterifying a pectin comprising treating the pectin with a recombinant enzyme comprising any one of the amino acid sequences shown as SEQ.I.D. No.1 or SEQ.I.D. No. 2, or a variant, derivative or homologue thereof, including combinations thereof.

10

According to a third aspect of the present invention there is provided a recombinant enzyme comprising any one of the amino acid sequences shown as SEQ.I.D. No.1 or SEQ.I.D. No. 2, or a variant, derivative or homologue thereof, including combinations thereof.

15

According to a fourth aspect of the present invention there is provided a nucleotide sequence coding for the recombinant enzyme according to the present invention, wherein the nucleotide sequence comprises any one of the sequences shown as SEQ. I.D. No. 3 or SEQ. I.D. No.4. or a variant, derivative or homologue thereof.

20

According to a fifth aspect of the present invention there is provided a nucleotide sequence coding for the recombinant enzyme according to the present invention wherein the nucleotide sequence is obtainable from NCIMB 40749 or NCIMB 40750, or a variant, derivative or homologue thereof.

25

According to a sixth aspect of the present invention there is provided a construct expressing or comprising the recombinant enzyme according to the present invention or a nucleotide sequence according to the present invention.

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According to a seventh aspect of the present invention there is provided a vector expressing or comprising a construct according to the present invention or the recombinant enzyme according to the present invention or a nucleotide sequence according to the present invention.

5

According to an eighth aspect of the present invention there is provided a combination of constructs comprising at least a first construct expressing or comprising the recombinant enzyme according to the present invention or a nucleotide sequence according to the present invention; and a second construct comprising a gene of interest (GOI) and a promoter.

10

According to a ninth aspect of the present invention there is provided a cell, tissue or organ expressing or comprising a vector according to the present invention or a construct according to the present invention or the recombinant enzyme according to the present invention or a nucleotide sequence according to the present invention or a combination of constructs according to the present invention.

15

According to a tenth aspect of the present invention there is provided a transgenic organism expressing or comprising any of the afore-mentioned aspects of the present invention.

20

According to an eleventh aspect of the present invention there is provided NCIMB 40749 or NCIMB 40750.

25

According to a twelfth aspect of the present invention there is provided a recombinant PME enzyme which is immunologically reactive with an antibody raised against a purified recombinant enzyme according to the third aspect of the present invention, but not a tomato PME enzyme.

30

According to a thirteenth aspect of the present invention there is provided the use of the recombinant enzyme according to the present invention to reduce the number of ester groups of a pectin.

According to a fourteenth aspect of the present invention there is provided the use of the recombinant enzyme according to the present invention to de-esterify pectin in a block-wise manner.

5 According to a fifteenth aspect of the present invention there is provided the use of the recombinant enzyme according to the present invention to affect the calcium sensitivity of pectin.

10 According to a sixteenth aspect of the present invention there is provided the use of the recombinant enzyme according to the present invention to esterify pectin containing free carboxyl groups.

15 According to a seventeenth aspect of the present invention there is provided a pectin obtained by use of the recombinant enzyme according to the present invention, such as by the method according to the present invention.

20 According to an eighteenth aspect of the present invention there is provided a combination of enzymes comprising a recombinant enzyme according to the present invention and a fungal PME and/or an other pectin degrading enzyme (e.g. pectin lyase).

25 Other aspects of the present invention include the use of a pectin according to the present invention to prepare a foodstuff; and the use of a pectin according to the present invention to stabilise a protein in an acidic environment without adversely affecting the viscosity of the environment; and a recombinant nucleotide sequence coding for the enzyme according to the present invention.

30 Thus, the present invention relates to a new use of de-esterified pectins. The present invention also relates to a new recombinant PME to prepare such pectins.

Some of the key advantages of the present invention are that the de-esterified pectin of the present invention offers stability to proteins in an acidic environment without adversely affecting the viscosity of the environment.

5 A preferred embodiment of the first aspect of the present invention is a process comprising adding to an acidic environment, which contains at least one protein, a block-wise enzymatically de-esterified pectin prepared by use of recombinant DNA techniques, wherein the pectin is a high ester pectin.

10 The term "a block-wise enzymatically de-esterified pectin prepared by use of recombinant DNA techniques" means a pectin containing block-wise de-esterified groups wherein the pectin is prepared by treating (e.g. contacting) a pectin containing esterified groups with an enzyme that has been prepared by use of recombinant DNA techniques.

15 Some of the key advantages of this preferred aspect of the present invention are that the de-esterified pectin can be prepared with ease and with a relative degree of consistency. In this regard, the recombinant PME itself can be prepared fairly simply and easily and, also, to a high degree of homogeneity. This in turn, and unlike the
20 prior art PME preparations, means that the resultant PME activity is more consistent and homogeneous so enables the overall de-esterification process to be more controlled.

The use of a block-wise enzymatically de-esterified pectin - which is preferably
25 prepared by use of recombinant DNA techniques - in the process of the present invention for stabilising at least one protein in an acidic environment is of benefit as it allows proteins such as whey and milk proteins (such as casein) to be stable in acidic solutions. This is of importance for the drinks market, such as skimmed milk, fruit juices and whey protein drinks, wherein before it was only possible to retain the
30 flavour of the key proteins under fairly high acidic conditions - such as pH 4.2 - if high amounts of stabiliser were present.

We have now found that for some applications small amounts of the de-esterified pectin according to the present invention can be employed. At these low levels, the de-esterified pectin according to the present invention not only acts as a stabiliser but also it does not have an adverse effect on the final product.

5

If desired, the use of the de-esterified pectin of the present invention would enable food manufacturers to increase the pH of foods, such as drinks. In this regard, in some cases the less acidic nature of the drinks may make them more palatable for people, especially infants. Thus, in contrast to the prior art processes, it is now possible to retain the flavour of those proteins at pH conditions higher than 4.2, such as up to pH 5.5 (such as pH 5.2) by use of the block-wise enzymatically de-esterified pectin, particularly the block-wise enzymatically de-esterified pectin prepared by use of recombinant DNA techniques.

10

In addition, it is believed that even under low pH conditions, such as pH 4.2 or less, the block-wise enzymatically de-esterified pectin - particularly the block-wise enzymatically de-esterified pectin prepared by use of recombinant DNA techniques - stabilises the protein(s) more than the prior art stabilisers that are used for those pH conditions.

20

A further advantage is that the recombinant PME is capable of producing a substantially homogeneous block-wise de-esterified pectin. By this we mean that substantially all of the pectin chains comprise at least two adjacent de-esterified carboxyl groups. However, for some applications it may not be necessary to prepare or use such a substantially homogeneous block-wise de-esterified pectin.

25

Without wishing to be bound by theory it is believed that the block-wise enzymatically de-esterified pectin - particularly that prepared by use of recombinant DNA techniques - stabilises the protein(s) by surrounding the protein(s) in a blanket of negative charges, thus forming a stable emulsion.

30

The recombinant PME enzyme of the present invention is useful for blockwise de-esterifying pectins when the pectins are contacted with the recombinant enzyme in a substantially aqueous medium. In some instances, de-esterifying pectins can increase the calcium ion sensitivity of a pectin - which in turn may be advantageous.

5

Alternatively, the recombinant PME enzyme of the present invention is useful for esterifying pectins when the pectins are contacted with the recombinant enzyme in a substantially non-aqueous medium, such as in the presence of methanol or in the presence of high concentrations of ammonium sulphate. This aspect is advantageous if, for example, it is desirable to reduce the calcium sensitivity of a pectin.

10

This method of esterifying pectins is advantageous because it obviates the need for the high temperature and methanol esterification conditions associated with the prior art processes. Thus, the present invention also includes the use of that esterified pectin in the preparation of a foodstuff, as well as the pectin *per se*.

15

In accordance with the present invention, the de-esterified pectin of the present invention is advantageous for the preparation of a foodstuff.

20

Typical foodstuffs include dairy products, meat products, poultry products, fish products and bakery products. Preferably, the foodstuff is a beverage.

The de-esterified pectin of the present invention is also advantageous for use as a stabiliser and/or viscosity modifier in the preparation of pharmaceuticals, pharmaceutical appliances, cosmetics and cosmetic appliances.

25

Preferably the acidic environment is an aqueous solution.

Preferably the aqueous solution is a beverage.

30

Preferably the beverage is a drinking yoghurt, a fruit juice or a beverage comprising whey protein.

Preferably the protein is derived or derivable from dairy products, such as milk or cheese.

Preferably the protein is casein or whey protein.

Preferably the acidic environment has a pH of from about 3.5 to about 5.5.

Preferably the acidic environment has a pH of from 4 to about 5.5.

Preferably, wherein the acidic environment has a pH of about 4.

Preferably the block-wise enzymatically de-esterified pectin, particularly that prepared by recombinant DNA techniques, is a high ester pectin containing about 80% ester groups or less (i.e. a degree of esterification (DE) of 80% or less), preferably about 75% ester groups or less (i.e. a DE of about 75% or less). In this regard, the ratio of free carboxyl groups to esterified carboxyl groups on the pectin is from 1:1 to 1:4, preferably from 1:2 to 1:3.

Preferably, the block-wise enzymatically de-esterified pectin contains about 76% ester groups.

In some instances, preferably the block-wise enzymatically de-esterified pectin is sensitive to Ca^{2+} ions. Calcium sensitivity can be determined by following the Protocol as mentioned in the Examples.

More preferably, however, the block-wise enzymatically de-esterified pectin, particularly that prepared by recombinant DNA techniques, is insensitive to Ca^{2+} ions. Calcium insensitivity can be determined by following the Protocol as mentioned in the Examples.

Preferably the block-wise enzymatically de-esterified pectin has a high molecular weight.

Typically, the molecular weight is between from about 50 KD to about 150 KD.

5 Preferably the block-wise enzymatically de-esterified pectin is prepared by treating a pectin with a recombinant pectin methyl esterase that de-esterifies two or more adjacent galacturonic acid residues of the pectin on at least substantially all of the pectin chains.

10 Preferably the recombinant pectin methyl esterase is derived from a PME obtainable from a plant.

The term "derived from a PME obtainable from a plant" means that the recombinant PME has a sequence similar to that of a PME that is obtainable from a plant, providing the recombinant PME can de-esterify pectin in a block-wise manner.

15 Preferably the plant is a fruit.

Preferably the fruit is a citrus fruit.

20 Preferably the citrus fruit is an orange.

Preferably the recombinant pectin methyl esterase is derived from a PME obtainable from the lamella or albedo of an orange. For reference, a cross-section of a typical citrus fruit is shown in Figure 1 where the lamella and albedo are indicated. The term "derived from a PME obtainable from the lamella or albedo of an orange" means that the recombinant PME has a sequence similar to that of a PME that is obtainable from the lamella or albedo of an orange, providing the recombinant PME can de-esterify pectin in a block-wise manner.

30 Preferably, the block-wise enzymatically de-esterified pectin is prepared by treating a pectin with a recombinant enzyme comprising any one of the amino acid sequences shown as SEQ.I.D. No.1 or SEQ.I.D. No. 2, or a variant, derivative or homologue thereof, including combinations thereof.

Preferably, the block-wise enzymatically de-esterified pectin is prepared by treating a pectin with a recombinant enzyme that is obtainable by expression of a nucleotide sequence comprising the nucleotide sequence shown as SEQ.I.D. No. 3 or SEQ.I.D. No. 4, or a variant, derivative or homologue thereof, or combinations thereof.

5

Preferably, the block-wise enzymatically de-esterified pectin is prepared by treating a pectin with a recombinant enzyme that is obtainable by expression of the PME coding sequence contained in NCIMB 40749 or NCIMB 40750, or a variant, derivative or homologue thereof, or combinations thereof.

10

Preferably, the block-wise enzymatically de-esterified pectin is prepared by treating the pectin with the recombinant pectin methyl esterase in the presence of sodium ions.

15

Preferably, the block-wise enzymatically de-esterified pectin prepared by recombinant DNA techniques is prepared by treating the pectin with the recombinant pectin methyl esterase in the presence of NaCl, NaNO₃ or Na₂SO₄.

20

Preferably the recombinant enzyme comprises all of the sequence shown as SEQ.I.D. No. 1 or SEQ.I.D. No. 2.

Preferably the recombinant enzyme is expressed by a nucleotide sequence that comprises all of the sequence shown as SEQ.I.D. No. 3 or SEQ.I.D. No. 4.

25

The present invention also covers sequences that are complementary to the aforementioned sequences.

30

The term "pectin" includes pectin in its normal sense, as well as fractionates and derivatives thereof, as well as modified pectins (e.g. chemically modified pectins and enzymatically modified pectins).

Preferably, the pectin is not a pectin that has been prior treated with the enzyme polygalacturonase to substantially reduce the length of the pectin backbone.

5 The terms "variant", "homologue" or "fragment" in relation to the recombinant enzyme of the present invention include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acid from or to the sequence providing the resultant amino acid sequence has PME activity, preferably having at least the same activity of a recombinant enzyme comprising any one or more of the sequences shown as SEQ I.D. No.s 1 and 2. In particular, the term
10 "homologue" covers homology with respect to structure and/or function providing the resultant recombinant enzyme has PME activity. With respect to sequence homology (i.e. similarity), preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequences shown in the attached sequence listings. More preferably there is at least 95%, more preferably at least 98%,
15 homology to the sequences shown in the attached sequence listings.

The terms "variant", "homologue" or "fragment" in relation to the nucleotide sequence coding for the recombinant enzyme of the present invention include any substitution of, variation of, modification of, replacement of, deletion of or addition
20 of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence codes for a recombinant enzyme having PME activity, preferably having at least the same activity of a recombinant enzyme comprising any one or more of the sequences shown as SEQ I.D. No.s 1 and 2. In particular, the term
25 "homologue" covers homology with respect to structure and/or function providing the resultant nucleotide sequence codes for a recombinant enzyme having PME activity. With respect to sequence homology (i.e. similarity), preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology. More preferably there is at least 95%, more preferably at least 98%, homology.

30 The above terms are synonymous with allelic variations of the sequences.

The term "complementary" means that the present invention also covers nucleotide sequences that can hybridise to the nucleotide sequences of the coding sequence.

5 The term "nucleotide" in relation to the present invention includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA for the coding sequence of the present invention.

10 The term "citrus fruit" means species of the genus *Citrus* and includes lime, lemon, orange, grapefruit, kumquat, pomelo, and mandarin. Preferably, it means orange.

15 The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes the nucleotide sequence according to the present invention or, the case of the combination of constructs, the GOI directly or indirectly attached to a promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the *Sh1*-intron or the ADH intron, intermediate the promoter and the nucleotide sequence of the present invention or the GOI. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. In each case, the terms do not cover the natural combination of the gene coding for the enzyme ordinarily associated with the wild type gene promoter and when they are both in their natural environment.

25 The construct may even contain or express a marker which allows for the selection of the genetic construct in, for example, a filamentous fungus, preferably of the genus *Aspergillus*, such as *Aspergillus niger*, or plants, such as potatoes, sugar beet etc., into which it has been transferred. Various markers exist which may be used, such as for example those encoding mannose-6-phosphate isomerase (especially for plants) or those markers that provide for antibiotic resistance - e.g. resistance to G418, hygromycin, bleomycin, kanamycin and gentamycin.

30 The term "vector" includes expression vectors and transformation vectors.

The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

5 The term "transformation vector" means a construct capable of being transferred from one species to another - such as from an *E.coli* plasmid to a filamentous fungus, preferably of the genus *Aspergillus*. It may even be a construct capable of being transferred from an *E.coli* plasmid to an *Agrobacterium* to a plant.

10 The term "tissue" includes tissue *per se* and organ.

The term "organism" in relation to the present invention includes any organism that could comprise the nucleotide sequence coding for the recombinant enzyme according to the present invention and/or products obtained therefrom, wherein a promoter can allow expression of the nucleotide sequence according to the present invention when
15 present in the organism.

Preferably the organism is a filamentous fungus, preferably of the genus *Aspergillus*, more preferably *Aspergillus niger*.

20 The term "transgenic organism" in relation to the present invention includes any organism that comprises the nucleotide sequence coding for the recombinant enzyme according to the present invention and/or products obtained therefrom, wherein the promoter can allow expression of the nucleotide sequence according to the present invention within the organism. Preferably the nucleotide sequence is incorporated in
25 the genome of the organism.

Preferably the transgenic organism is a filamentous fungus, preferably of the genus *Aspergillus*, more preferably *Aspergillus niger*.

30 Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, a promoter, the nucleotide sequence coding for the recombinant enzyme according to the present invention, constructs

according to the present invention (including combinations thereof), vectors according to the present invention, plasmids according to the present invention, cells according to the present invention, tissues according to the present invention or the products thereof.

5

The term "transgenic organism" does not cover the native nucleotide coding sequence according to the present invention in its natural environment when it is under the control of its native promoter which is also in its natural environment. In addition, the present invention does not cover the native enzyme according to the present invention when it is in its natural environment and when it has been expressed by its native nucleotide coding sequence which is also in its natural environment and when that nucleotide sequence is under the control of its native promoter which is also in its natural environment.

10

The transformed cell or organism could prepare acceptable quantities of the desired compound which would be easily retrievable from, the cell or organism.

15

Preferably the construct of the present invention comprises the nucleotide sequence of the present invention and a promoter.

20

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Monod theory of gene expression.

In one aspect, the nucleotide sequence according to the present invention is under the control of a promoter that may be a cell or tissue specific promoter. If, for example, the organism is a plant then the promoter can be one that affects expression of the nucleotide sequence in any one or more of tuber, stem, sprout, root and leaf tissues.

25

By way of example, the promoter for the nucleotide sequence of the present invention can be the α -Amy 1 promoter (otherwise known as the Amy 1 promoter, the Amy 637 promoter or the α -Amy 637 promoter) as described in our co-pending UK patent application No. 9421292.5 filed 21 October 1994. Alternatively, the promoter for

30

the nucleotide sequence of the present invention can be the α -Amy 3 promoter (otherwise known as the Amy 3 promoter, the Amy 351 promoter or the α -Amy 351 promoter) as described in our co-pending UK patent application No. 9421286.7 filed 21 October 1994.

5

The promoter could additionally include features to ensure or to increase expression in a suitable host. For example, the features can be conserved regions such as a Pribnow Box or a TATA box. The promoter may even contain other sequences to affect (such as to maintain, enhance, decrease) the levels of expression of the nucleotide sequence of the present invention or, in the case of the combination of constructs, the GOI. For example, suitable other sequences include the *Shl*-intron or an ADH intron. Other sequences include inducible elements - such as temperature, chemical, light or stress inducible elements. Also, suitable elements to enhance transcription or translation may be present. An example of the latter element is the TMV 5' signal sequence (see Sleat Gene 217 [1987] 217-225; and Dawson Plant Mol. Biol. 23 [1993] 97).

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In addition the present invention also encompasses combinations of promoters and/or nucleotide sequences coding for proteins or recombinant enzymes and/or elements.

25

The present invention also encompasses the use of promoters to express a nucleotide sequence coding for the recombinant enzyme according to the present invention or the GOI, wherein a part of the promoter is inactivated but wherein the promoter can still function as a promoter. Partial inactivation of a promoter in some instances is advantageous. In particular, with the Amy 351 promoter mentioned earlier it is possible to inactivate a part of it so that the partially inactivated promoter expresses the nucleotide of the present invention or a GOI in a more specific manner such as in just one specific tissue type or organ.

30

The term "inactivated" means partial inactivation in the sense that the expression pattern of the promoter is modified but wherein the partially inactivated promoter still functions as a promoter. However, as mentioned above, the modified promoter is

capable of expressing the nucleotide of the present invention or a GOI in at least one (but not all) specific tissue of the original promoter. One such promoter is the Amy 351 promoter described above. Examples of partial inactivation include altering the folding pattern of the promoter sequence, or binding species to parts of the nucleotide sequence, so that a part of the nucleotide sequence is not recognised by, for example, RNA polymerase. Another, and preferable, way of partially inactivating the promoter is to truncate it to form fragments thereof. Another way would be to mutate at least a part of the sequence so that the RNA polymerase can not bind to that part or another part. Another modification is to mutate the binding sites for regulatory proteins for example the CreA protein known from filamentous fungi to exert carbon catabolite repression, and thus abolish the catabolite repression of the native promoter.

The term "GOI" with reference to the combination of constructs according to the present invention means any gene of interest. A GOI can be any nucleotide that is either foreign or natural to the organism (e.g. filamentous fungus, preferably of the genus *Aspergillus*, or a plant) in question. Typical examples of a GOI include genes encoding for proteins and enzymes that modify metabolic and catabolic processes. The GOI may code for an agent for introducing or increasing pathogen resistance. The GOI may even be an antisense construct for modifying the expression of natural transcripts present in the relevant tissues. The GOI may even code for a non-native protein of a filamentous fungus, preferably of the genus *Aspergillus*, or a compound that is of benefit to animals or humans.

Examples of GOIs include other pectinases, pectin depolymerases, polygalacturonases, pectate lyases, pectin lyases, rhamno-galacturonases, hemicellulases, endo- β -glucanases, arabinases, or acetyl esterases, or combinations thereof, as well as antisense sequences thereof.

The GOI may be a protein giving nutritional value to a food or crop. Typical examples include plant proteins that can inhibit the formation of anti-nutritive factors and plant proteins that have a more desirable amino acid composition (e.g. a higher

lysine content than a non-transgenic plant). The GOI may even code for an enzyme that can be used in food processing such as chymosin, thaumatin and α -galactosidase. The GOI can be a gene encoding for any one of a pest toxin, an antisense transcript such as that for patatin or α -amylase, ADP-glucose pyrophosphorylase (e.g. see EP-A-0455316), a protease antisense, a glucanase or genomic PME.

The GOI may even code for an intron of a particular enzyme but wherein the intron can be in sense or antisense orientation. In the latter instance, the particular enzyme could be genomic PME. Antisense expression of genomic exon or intron sequences as the GOI would mean that the natural PME expression would be reduced or eliminated but wherein the recombinant PME expression would not be affected. This is particularly true for antisense intron or sense intron expression.

The GOI can be the nucleotide sequence coding for the α -amylase enzyme which is the subject of our co-pending UK patent application 9413439.2 filed on 4 July 1994. The GOI can be the nucleotide sequence coding for the α -amylase enzyme which is the subject of our co-pending UK patent application 9421290.9 filed on 21 October 1994. The GOI can be any of the nucleotide sequences coding for the ADP-glucose pyrophosphorylase enzymes which are the subject of our co-pending PCT patent application PCT/EP94/01082 filed 7 April 1994. The GOI can be any of the nucleotide sequences coding for the α -glucan lyase enzyme which are described in our co-pending PCT patent application PCT/EP94/03397 filed 15 October 1994.

The host organism can be a prokaryotic or a eukaryotic organism. Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*. Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook *et al* (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press). If a prokaryotic host is used then the gene may need to be suitably modified before transformation - such as by removal of introns.

As mentioned above, a preferred host organism is of the genus *Aspergillus*, such as *Aspergillus niger*.

A transgenic *Aspergillus* according to the present invention can be prepared by following the teachings of Rambosek, J. and Leach, J. 1987 (Recombinant DNA in filamentous fungi: Progress and Prospects. CRC Crit. Rev. Biotechnol. 6:357-393), Davis R.W. 1994 (Heterologous gene expression and protein secretion in *Aspergillus*. In: Martinelli S.D., Kinghorn J.R.(Editors) *Aspergillus: 50 years on. Progress in industrial microbiology* vol 29. Elsevier Amsterdam 1994. pp 525-560), Ballance, D.J. 1991 (Transformation systems for Filamentous Fungi and an Overview of Fungal Gene structure. In: Leong, S.A., Berka R.M. (Editors) *Molecular Industrial Mycology. Systems and Applications for Filamentous Fungi*. Marcel Dekker Inc. New York 1991. pp 1-29) and Turner G. 1994 (Vectors for genetic manipulation. In: Martinelli S.D., Kinghorn J.R.(Editors) *Aspergillus: 50 years on. Progress in industrial microbiology* vol 29. Elsevier Amsterdam 1994. pp. 641-666). However, the following commentary provides a summary of those teachings for producing transgenic *Aspergillus* according to the present invention.

For almost a century, filamentous fungi have been widely used in many types of industry for the production of organic compounds and enzymes. For example, traditional Japanese koji and soy fermentations have used *Aspergillus sp.* Also, in this century *Aspergillus niger* has been used for production of organic acids particular citric acid and for production of various enzymes for use in industry.

There are two major reasons why filamentous fungi have been so widely used in industry. First filamentous fungi can produce high amounts of extracellular products, for example enzymes and organic compounds such as antibiotics or organic acids. Second filamentous fungi can grow on low cost substrates such as grains, bran, beet pulp etc. The same reasons have made filamentous fungi attractive organisms as hosts for heterologous expression according to the present invention.

In order to prepare the transgenic *Aspergillus*, expression constructs are prepared by inserting the nucleotide sequence according to the present invention (or even the GOI) into a construct designed for expression in filamentous fungi.

Several types of constructs used for heterologous expression have been developed. These constructs preferably contain a promoter which is active in fungi. Examples of promoters include a fungal promoter for a highly expressed extracellular enzyme, such as the glucoamylase promoter or the α -amylase promoter. The nucleotide sequence according to the present invention (or even the GOI) can be fused to a signal sequence which directs the protein encoded by the nucleotide sequence according to the present invention (or even the GOI) to be secreted. Usually a signal sequence of fungal origin is used. A terminator active in fungi ends the expression system.

Another type of expression system has been developed in fungi where the nucleotide sequence according to the present invention (or even the GOI) can be fused to a smaller or a larger part of a fungal gene encoding a stable protein. This can stabilize the protein encoded by the nucleotide sequence according to the present invention (or even the GOI). In such a system a cleavage site, recognized by a specific protease, can be introduced between the fungal protein and the protein encoded by the nucleotide sequence according to the present invention (or even the GOI), so the produced fusion protein can be cleaved at this position by the specific protease thus liberating the protein encoded by the nucleotide sequence according to the present invention (or even the GOI). By way of example, one can introduce a site which is recognized by a KEX-2 like peptidase found in at least some *Aspergilli*. Such a fusion leads to cleavage *in vivo* resulting in protection of the expressed product and not a larger fusion protein.

Heterologous expression in *Aspergillus* has been reported for several genes coding for bacterial, fungal, vertebrate and plant proteins. The proteins can be deposited intracellularly if the nucleotide sequence according to the present invention (or even the GOI) is not fused to a signal sequence. Such proteins will accumulate in the cytoplasm and will usually not be glycosylated which can be an advantage for some bacterial proteins. If the nucleotide sequence according to the present invention (or even the GOI) is equipped with a signal sequence the protein will accumulate extracellularly.

With regard to product stability and host strain modifications, some heterologous proteins are not very stable when they are secreted into the culture fluid of fungi. Most fungi produce several extracellular proteases which degrade heterologous proteins. To avoid this problem special fungal strains with reduced protease
5 production have been used as host for heterologous production.

For the transformation of filamentous fungi, several transformation protocols have been developed for many filamentous fungi (Ballance 1991, *ibid*). Many of them are based on preparation of protoplasts and introduction of DNA into the protoplasts
10 using PEG and Ca^{2+} ions. The transformed protoplasts then regenerate and the transformed fungi are selected using various selective markers. Among the markers used for transformation are a number of auxotrophic markers such as *argB*, *trpC*, *niaD* and *pyrG*, antibiotic resistance markers such as benomyl resistance, hygromycin resistance and phleomycin resistance. A commonly used transformation marker is the
15 *amdS* gene of *A. nidulans* which in high copy number allows the fungus to grow with acrylamide as the sole nitrogen source.

In another embodiment the transgenic organism can be a yeast. In this regard, yeast have also been widely used as a vehicle for heterologous gene expression. The
20 species *Saccharomyces cerevisiae* has a long history of industrial use, including its use for heterologous gene expression. Expression of heterologous genes in *Saccharomyces cerevisiae* has been reviewed by Goodey *et al* (1987, Yeast Biotechnology, D R Berry *et al*, eds, pp 401-429, Allen and Unwin, London) and by King *et al* (1989, Molecular and Cell Biology of Yeasts, E F Walton and G T
25 Yarronton. eds, pp 107-133, Blackie, Glasgow).

For several reasons *Saccharomyces cerevisiae* is well suited for heterologous gene expression. First, it is non-pathogenic to humans and it is incapable of producing certain endotoxins. Second, it has a long history of safe use following centuries of
30 commercial exploitation for various purposes. This has led to wide public acceptability. Third, the extensive commercial use and research devoted to the organism has resulted in a wealth of knowledge about the genetics and physiology as

well as large-scale fermentation characteristics of *Saccharomyces cerevisiae*.

5 A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

10 Several types of yeast vectors are available, including integrative vectors, which require recombination with the host genome for their maintenance, and autonomously replicating plasmid vectors.

15 In order to prepare the transgenic *Saccharomyces*, expression constructs are prepared by inserting the nucleotide sequence of the present invention into a construct designed for expression in yeast. Several types of constructs used for heterologous expression have been developed. The constructs contain a promoter active in yeast fused to the nucleotide sequence of the present invention, usually a promoter of yeast origin, such as the GAL1 promoter, is used. Usually a signal sequence of yeast origin, such as the sequence encoding the SUC2 signal peptide, is used. A terminator active in yeast ends the expression system.

20 For the transformation of yeast several transformation protocols have been developed. For example, a transgenic *Saccharomyces* according to the present invention can be prepared by following the teachings of Hinnen *et al* (1978, Proceedings of the National Academy of Sciences of the USA 75, 1929); Beggs, J D (1978, Nature, London, 275, 104); and Ito, H *et al* (1983, J Bacteriology 153, 163-168).

25 The transformed yeast cells are selected using various selective markers. Among the markers used for transformation are a number of auxotrophic markers such as LEU2, HIS4 and TRP1, and dominant antibiotic resistance markers such as aminoglycoside antibiotic markers, eg G418.

30

Another host organism is a plant.

Even though the enzyme and the nucleotide sequence coding therefor are not disclosed in EP-B-0470145 and CA-A-2006454, those two documents do provide some useful background commentary on the types of techniques that may be employed to prepare transgenic plants according to the present invention. Some of
5 these background teachings are now included in the following commentary.

The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material.
10

Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-
15 225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

Thus, in one aspect, the present invention relates to a vector system which carries a nucleotide sequence or construct according to the present invention and which is capable of introducing the nucleotide sequence or construct into the genome of an
20 organism, such as a plant.

The vector system may comprise one vector, but it can comprise two vectors. In the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An et al.
25 (1980), Binary Vectors, *Plant Molecular Biology Manual A3*, 1-19.

One extensively employed system for transformation of plant cells with a given promoter or nucleotide sequence or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* An et al.
30 (1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208.

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above. A non-limiting example of such a Ti plasmid is pGV3850.

5 The nucleotide sequence or construct of the present invention should preferably be inserted into the Ti-plasmid between the terminal sequences of the T-DNA or adjacent a T-DNA sequence so as to avoid disruption of the sequences immediately surrounding the T-DNA borders, as at least one of these regions appear to be essential for insertion of modified T-DNA into the plant genome.

10

As will be understood from the above explanation, if the organism is a plant, then the vector system of the present invention is preferably one which contains the sequences necessary to infect the plant (e.g. the *vir* region) and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic
15 construct. Preferably, the vector system is an *Agrobacterium tumefaciens* Ti-plasmid or an *Agrobacterium rhizogenes* Ri-plasmid or a derivative thereof, as these plasmids are well-known and widely employed in the construction of transgenic plants, many vector systems exist which are based on these plasmids or derivatives thereof.

20 In the construction of a transgenic plant the nucleotide sequence or construct of the present invention may be first constructed in a microorganism in which the vector can replicate and which is easy to manipulate before insertion into the plant. An example of a useful microorganism is *E. coli.*, but other microorganisms having the above properties may be used. When a vector of a vector system as defined above has been
25 constructed in *E. coli.* it is transferred, if necessary, into a suitable *Agrobacterium* strain, e.g. *Agrobacterium tumefaciens*. The Ti-plasmid harbouring the nucleotide sequence or construct of the invention is thus preferably transferred into a suitable *Agrobacterium* strain, e.g. *A. tumefaciens*, so as to obtain an *Agrobacterium* cell harbouring the nucleotide sequence or construct of the invention, which DNA is
30 subsequently transferred into the plant cell to be modified.

As reported in CA-A-2006454, a large amount of cloning vectors are available which contain a replication system in *E. coli* and a marker which allows a selection of the transformed cells. The vectors contain for example pBR 322, the pUC series, the M13 mp series, pACYC 184 etc.

5

In this way, the nucleotide or construct of the present invention can be introduced into a suitable restriction position in the vector. The contained plasmid is used for the transformation in *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium and then harvested and lysed. The plasmid is then recovered. As a method of
10 analysis there is generally used sequence analysis, restriction analysis, electrophoresis and further biochemical-molecular biological methods. After each manipulation, the used DNA sequence can be restricted and connected with the next DNA sequence. Each sequence can be cloned in the same or different plasmid.

15

After each introduction method of the desired promoter or construct or nucleotide sequence according to the present invention in the plants the presence and/or insertion of further DNA sequences may be necessary. If, for example, for the transformation the Ti- or Ri-plasmid of the plant cells is used, at least the right boundary and often however the right and the left boundary of the Ti- and Ri-plasmid T-DNA, as
20 flanking areas of the introduced genes, can be connected. The use of T-DNA for the transformation of plant cells has been intensively studied and is described in EP-A-120516; Hoekema, in: The Binary Plant Vector System Offset-drukkerij Kanter B.B., Alblasterdam, 1985, Chapter V; Fraley, *et al.*, Crit. Rev. Plant Sci., 4:1-46; and An *et al.*, EMBO J. (1985) 4:277-284.

25

Direct infection of plant tissues by *Agrobacterium* is a simple technique which has been widely employed and which is described in Butcher D.N. *et al.* (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208. For further teachings on this topic see Potrykus (Annu Rev Plant Physiol Plant
30 Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). With this technique, infection of a plant may be done on a certain part or tissue of the plant, i.e. on a part of a leaf, a root, a stem or another part of the

plant.

Typically, with direct infection of plant tissues by *Agrobacterium* carrying the promoter and/or the GOI, a plant to be infected is wounded, e.g. by cutting the plant with a razor or puncturing the plant with a needle or rubbing the plant with an abrasive. The wound is then inoculated with the *Agrobacterium*. The inoculated plant or plant part is then grown on a suitable culture medium and allowed to develop into mature plants.

When plant cells are constructed, these cells may be grown and maintained in accordance with well-known tissue culturing methods such as by culturing the cells in a suitable culture medium supplied with the necessary growth factors such as amino acids, plant hormones, vitamins, etc. Regeneration of the transformed cells into genetically modified plants may be accomplished using known methods for the regeneration of plants from cell or tissue cultures, for example by selecting transformed shoots using an antibiotic and by subculturing the shoots on a medium containing the appropriate nutrients, plant hormones, etc.

Further teachings on plant transformation may be found in EP-A-0449375.

In summation, the present invention provides a process of stabilising at least one protein in an acidic environment comprising contacting the protein with a block-wise enzymatically de-esterified pectin, in particular a block-wise enzymatically de-esterified pectin prepared by recombinant DNA techniques.

In addition the present invention provides a recombinant enzyme useful in that process.

A preferred embodiment of the present invention relates to a process of stabilising at least one protein in an acidic environment comprising contacting the protein with a block-wise enzymatically de-esterified pectin prepared by recombinant DNA techniques, wherein the block-wise enzymatically de-esterified pectin is prepared by

use of a recombinant enzyme, and wherein the recombinant enzyme comprises any one of the amino acid sequences shown as SEQ.I.D. No.1 or SEQ.I.D. No. 2 or a variant, derivative or homologue thereof, including combinations thereof, and has the following characteristics:

5

1. a molecular weight of from about 36 kD to about 64 kD;
2. a pH optimum of pH 7 - 8 when measured with 0.5% lime pectin in 0.15 M NaCl;
3. a temperature optimum of at least 50°C;
- 10 4. a temperature stability in the range of from 10°- at least 40°C;
5. a K_m value of 0.07 %;
6. an activity maximum at levels of about 0.25 M NaCl;
7. an activity maximum at levels of about 0.2 M Na_2SO_4 ; and
8. an activity maximum at levels of about 0.3 M NaNO_3 .

15

Another preferred embodiment of the present invention relates to a method of block-wise enzymatically de-esterifying a pectin comprising treating the pectin with a recombinant enzyme comprising any one of the amino acid sequences shown as SEQ.I.D. No.1 or SEQ.I.D. No. 2, or a variant, derivative or homologue thereof, including combinations thereof, wherein the recombinant enzyme has the following characteristics:

20

1. a molecular weight of from about 36 kD to about 64 kD;
2. a pH optimum of pH 7 - 8 when measured with 0.5% lime pectin in 0.15 M NaCl;
- 25 3. a temperature optimum of at least 50°C;
4. a temperature stability in the range of from 10°- at least 40°C;
5. a K_m value of 0.07 %;
6. an activity maximum at levels of about 0.25 M NaCl;
- 30 7. an activity maximum at levels of about 0.2 M Na_2SO_4 ; and
8. an activity maximum at levels of about 0.3 M NaNO_3 .

Another preferred embodiment of the present invention relates to a recombinant enzyme comprising any one of the amino acid sequences shown as SEQ.I.D. No.1 or SEQ.I.D. No. 2, or a variant, derivative or homologue thereof, including combinations thereof, wherein the recombinant enzyme has the following characteristics:

1. a molecular weight of from about 36 kD to about 64 kD;
2. a pH optimum of pH 7 - 8 when measured with 0.5% lime pectin in 0.15 M NaCl;
- 10 3. a temperature optimum of at least 50°C;
4. a temperature stability in the range of from 10° - at least 40°C;
5. a K_m value of 0.07 %;
6. an activity maximum at levels of about 0.25 M NaCl;
7. an activity maximum at levels of about 0.2 M Na_2SO_4 ; and
- 15 8. an activity maximum at levels of about 0.3 M NaNO_3 .

More preferred embodiments of the present invention relate to the afore-mentioned preferred process, method and recombinant enzyme, and wherein the recombinant enzyme has been expressed by a nucleotide sequence comprising the sequence shown as SEQ.I.No. 3 or SEQ.I.D. No. 4, or a variant, derivative or homologue thereof.

The following sample has been deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St Machar Drive, Aberdeen, Scotland, AB2 1RY, United Kingdom, on 6 July 1995: NCIMB 40749 (which corresponds to plasmid pO34).

The following sample has been deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St Machar Drive, Aberdeen, Scotland, AB2 1RY, United Kingdom, on 12 July: NCIMB 40750 (which corresponds to plasmid pO17).

Therefore, more preferred embodiments of the present invention relate to the aforementioned process, method and recombinant enzyme and wherein the recombinant enzyme is capable of being expressed by NCIMB 40749 or NCIMB 40750.

5 The present invention also provides an amino acid sequence comprising the sequence shown as SEQ.I.D. No. 5, or a variant, derivative or homologue thereof, suitable for use in imparting or increasing heat stability to a protein.

10 In addition, the present invention provides a nucleotide sequence comprising the sequence shown as SEQ.I.D. No. 6, or a variant, derivative or homologue thereof, suitable for use in expressing an amino acid sequence for imparting or increasing heat stability to a protein.

15 With respect to these last aspects, the above commentary for variants, derivatives, homologues, constructs, vectors, transformation of host organisms is equally applicable though of course the amino acid sequence and nucleotide sequence in this case affect the heat stability of proteins.

20 In this regard, it is believed that the amino acid sequence shown as SEQ.I.D. No. 5 is capable of imparting or increasing the heat stability of a protein, such as the recombinant PME of the present invention. The amino acid sequence may also increase or impart heat stability to other proteins, including PMEs from other sources.

25 The present invention will now be described only by way of example, in which reference is made to the following attached Figures:

Figure 1, which is a schematic diagram of a citrus fruit, such as an orange;

30 Figure 2, which is a SDS PAGE gel of the recombinant enzyme of the present invention;

Figure 3, which is a plot to determine pH optimum;

Figure 4, which is a plot to determine temperature optimum;

5 Figure 5, which is a plot to determine temperature stability;

Figure 6, which is a plot to determine the K_m value;

10 Figure 7, which is a plot to determine activity in the presence or absence of NaCl;

Figure 8, which is a plot to determine activity in the presence or absence of Na_2SO_4 ;

Figure 9, which is a plot to determine activity in the presence or absence of NaNO_3 ;

15 Figure 10, which is a plasmid map of pO17;

Figure 11, which is a plasmid map of pO34;

20 Figure 12, which is a diagrammatic representation of two genes;

Figure 13, which is a plasmid map of pJK10;

Figure 14, which is a plasmid map of pJK11;

25 Figure 15, which is a plasmid map of pJK12;

Figure 16, which is a plasmid map of pJK20;

Figure 17, which is a plasmid map of pJK21; and

30

Figure 18, which is a plasmid map of pJK22.

Figure 1 was referred to in the above description. The other Figures are discussed in the commentary below.

EXPERIMENTAL SECTION

5

MATERIALS AND METHODS FOR BIOCHEMISTRY

Plant material: Immature Spanish oranges of the *Navelina* variety class I without seeds, were used for isolation of PME. The oranges were peeled manually and the
10 peels were stored at -80°C.

Extraction of PME from orange peel

PME was purified according to the following procedure. All operations were
15 performed at 4°C. 600 g frozen orange peels were thawed and cut into minor pieces. They were then homogenized in a Warring blender for 2 min in 1200 ml buffer (100 mM Na-succinate pH 6.2, 1 mM DTT). 36 g solid NaCl was added to the homogenate to reach an end-concentration of 3% (w/v) in order to isolate membrane bound proteins (Versteeg et al. (1978) Lebensmittel.-Wiss. u. Technol., 11: 267-274). After
20 2 hours incubation with gently stirring at 4°C the suspension was filtered through nylon mesh and the filtrate was centrifuged at 10,000 rpm for 20 min to remove insoluble residues.

The supernatant was then fractionated using $(\text{NH}_4)_2\text{SO}_4$ precipitation. The supernatant
25 was first precipitated with 30% $(\text{NH}_4)_2\text{SO}_4$ under slowly stirring for 30 min. After centrifugation at 20,000 rpm for 10 min the supernatant was further precipitated with 60% $(\text{NH}_4)_2\text{SO}_4$ for 30 min. The suspension was centrifuged as before and the precipitate was resuspended in 50 ml 50 mM MES, 1 mM DTT pH 6.8 and dialysed against the same buffer over night.

30

Chromatography

The dialysed sample was further separated by cation exchange chromatography. A 40-50 ml sample was applied to a CM-Sepharose™ CL-6B (1.5 x 15 cm) in two rounds with a 30 min wash between the rounds with buffer A: 50 mM MES, 1 mM DTT pH 6.8. After washing off the unbound proteins with buffer A the bound proteins were eluted with an increasing NaCl gradient from 0 - 0.4 M NaCl in total 500 ml. The flow was 25 ml/h and fractions of 8.33 ml were collected. The protein absorption profile was measured at 280 nm.

10

All fractions were analysed for PME activity and protein. The protein content was measured spectrophotometrically with the BioRad method.

The fractions containing PME activity were pooled and concentrated by press dialysis using Amicon filter system. Buffer exchange to 50 mM Tris, 1 mM DTT, 0.1 M NaCl pH 7 was done on the same system.

9 ml concentrated PME sample was then applied to a Sephacryl™ S-200 (2.6 x 70 cm) gel filtration column. The column was equilibrated with 50 mM Tris, 1 mM DTT, 0.1 M NaCl pH 7. The flow was 40 ml/h and fractions of 5.33 ml were collected. The fractions containing PME activity were pooled and concentrated.

20

Enzyme activity

PME catalyses the cleavage of methylester groups from pectin. During the purification steps PME was detected by a fast method using methyl red indicator test. Due to cleavage of methyl groups from galacturonic acid residues in the pectin chain, carboxyl groups were formed and the pH drops in the assay. The pH indicator - methyl red - changes colour at pH drop from yellow (pH 6.2) to pink (pH 4.2). The assay contained 1 ml 0.5 % Grindsted™ Pectin 1450 (DE 70%) (supplied by Danisco Ingredients, Danisco A/S) solubilized in 0.15 M NaCl pH 7 and 125 µl sample. The samples which showed positive methyl red test after 10 min incubation at 30°C were

30

then further measured by the titration method (Versteeg et al (1978) Lebensmittel.-Wiss. u. Technol., 11: 267-274).

5 With the titration method the assay contained 10 ml 0.5 % lime pectin (Grindsted™ Pectin 1450 - supplied by Danisco Ingredients, Danisco A/S) solubilized in 0.15 M NaCl pH 6.8 and 10 - 100 µl sample. Titration was performed with 0.02 M NaOH and the reaction was measured at room temperature. An automatic titrator was used (Versteeg et al. (1978) Lebensmittel.-Wiss. u. Technol., 11: 267-274).

10 SDS-PAGE/Western blotting

The purity of the PME fraction was investigated by SDS-PAGE using Pharmacia PhastSystem™ with 10 - 15% SDS-gradient gels. Electrophoresis and silver staining of the proteins were done as described by the manuals from Pharmacia. For
15 determination of pI IEF 3-9 PhastSystem™ gels were used.

Immuno gel electrophoresis was used for characterisation of polyclonal antibodies raised against orange peel PME. The enzyme fractions were separated on SDS-PAGE and transferred to NC-paper by semi-dry blotting technique on a Semidry
20 transfer unit of the PhastSystem™. The NC-paper was incubated with the primer antibody diluted 1:50 and stained with the second antibody coupled to alkaline phosphatase (Dako A/S Glostrup, Denmark) used in a dilution of 1:1000.

Peptide mapping

25

PME was digested with either trypsin or endo-proteinase Lys-C from *Lysobacter enzymogenes* (both enzyme preparations were sequencing grade purchased from Boehringer Mannheim, Germany).

30

100 µg purified PME was carboxy methylated with iodoacetamide to protect the reduced SH-groups. Then the protein was cleaved with trypsin (4 µg/20-100 µl). The hydrolytic cleavage was performed at 40°C for 2 x 3 hrs. The reaction was

stopped with addition of 20 μ l TFA. After centrifugation at 15,000 rpm for 5 min the peptides were purified on a reverse-phase HPLC column (Vydac 10 C18 column). 2 x 500 μ l samples were applied. The peptides were eluted and separated with an increasing acetonitrile gradient from 0.05 - 0.35% in 60 min in 0.1% TFA. The peptides were collected manually in Eppendorf tubes.

For digestion with endo-proteinase Lys-C, freeze dried PME (0.1 mg) was dissolved in 50 μ l of 8 M urea, 0.4 M NH_4HCO_3 , pH 8.4. After overlay with N_2 and addition of 5 μ l of 45 mM DTT, the protein was denatured and reduced for 15 min at 50°C under N_2 . After cooling to room temperature, 5 μ l of 100 mM iodoacetamide was added for the cysteines to be derivatised for 15 min at room temperature in the dark under N_2 . Subsequently, 90 μ l of water and 5 μ g of endo-proteinase Lys-C in 50 μ l 50 mM tricine and 10 mM EDTA, pH 8.0, was added and the digestion was carried out for 24 hrs at 37°C under N_2 .

The resulting peptides were separated as described for trypsin digested peptides.

Selected peptides were further purified on a Devosil 3 C₁₈ RP-HPLC column 0.46x10 cm (Novo Nordisk, Denmark). The purified peptides were then applied on an amino acid sequencer, Applied Biosystems 476A, using pulsed-liquid fast cycles.

STUDY 1

During purification of PME 600 g frozen orange peels were homogenized and after precipitation with 30 - 60% $(\text{NH}_4)_2\text{SO}_4$ and dialysis the sample was applied to a cation exchange column (CM-Sepharose™ CL-6B). PME binds strongly to a cation exchange column material at pH 6.8 whereas most of the proteins do not bind to the column and so elute in the wash volume. With increasing NaCl gradient PME eluted into two peaks with the major activity in PME I (fraction 49-53) at a NaCl concentration of 0.25 M. A minor PME peak eluted at a lower concentration of NaCl (fraction 25-32). The further purification was only performed for PME I, which contained the highest activity.

After concentration the PME fraction was further purified using gel filtration chromatography (Sephacryl™ S-200 column). Fractions containing the highest PME activity were pooled and concentrated on amicon filter dialysis.

- 5 In total approximately 4 mg PME was obtained from 600 g orange peels which corresponds to a protein yield of 12%.

SDS-PAGE showed only one protein band in the purified PME fraction with a MW of 36,000 D (Figure 2). Isoelectric focusing of PME showed that the pI was > 9.

10

Characterization and kinetic data

Characterization of PME and optima determinations were all done with the titration method as described in Materials and Methods.

15

pH optimum of PME activity was measured with 0.5% lime pectin (Grindsted™ Pectin 1450 - supplied by Danisco Ingredients, Danisco A/S) in 0.15 M NaCl. The data are shown in Figure 3. The optimum was found around pH 7 - 8.

- 20 Temperature optimum was found at 50°C - see the data shown in Figure 4.

The heat (temperature) stability of PME was determined by incubating the enzyme sample in Eppendorf tubes at different temperatures for 15 min. After incubation the enzyme activity was measured by traditional assay by the titration method. The stability of the enzyme activity was between 10° - 40°C - see the data in Figure 5.

25

The affinity for lime pectin (Grindsted™ Pectin 1450 - supplied by Danisco Ingredients, Danisco A/S) was determined by Lineweaver Burk plot of different pectin concentration versus activity. The data are shown in Figure 6. The K_m was calculated from the curve to be 0.07 %.

30

We have also found that the PME could also de-esterify sugar beet pectin. Sugar beet pectin contains 60% galacturonic acid residues some of whose carboxyl groups are methylated (an approximate DE of 60%) and, in addition, some of the galacturonic acid groups are acetylated at C-2 and/or C-3. The PME activity was measured as described in Materials and Methods except that 1% sugar beet pectin solubilized in 0.15 M NaCl was used in the assay as the sugar beet pectin contained approx. 60% pectin. The results showed that the PME could de-esterify sugar beet pectin, even though the galacturonic acid residues are acetylated at the C-2/C-3 positions.

	Enzyme activity ($\mu\text{mol}/\text{min}/\text{ml}$)
0.5 % lime pectin (Grindsted TM Pectin 1450 - supplied by Danisco Ingredients, Danisco A/S)	387 80
1.0 % sugar beet pectin	

Further experiments showed that the PME of the present invention preferably requires NaCl for activity - see the data in Figure 7. The activity increases with increasing NaCl concentration, with an optimum at 0.25 M NaCl. Higher concentrations are reducing the activity compared to the maximal activity.

Further experiments showed that other salts e.g. Na_2SO_4 or NaNO_3 can substitute NaCl for PME activity. The data are shown in Figures 8 and 9. The optimum activity was found at 0.2 M Na_2SO_4 and 0.3 M NaNO_3 , respectively.

N-terminal analysis

5 Studies showed that the N-terminal sequence of native PME was blocked. De-blocking was achieved by treating PME blotted onto a PVDF membrane with anhydrous TFA for 4 min at 45°C in tubes. After evaporation of most of TFA the tubes were placed at 65°C for 4 hrs (Wellner, D. *et al.* (1990) Proc. Natl. Acad. Sci. 87: 1947-1949). The sequence obtained was SSSVTPNVVVAADSSGNFK and that N-acetylserine is the N-terminal residue of PME.

10 Immuno histo localization

For the preparation of tissue samples for immuno histo chemistry, thin slices of the middle part of mature fruit were fixed in 2% paraformaldehyde, 0.25% glutaraldehyde and 3% sucrose buffered with 0.05 M phosphate buffer pH 7. After 15 incubation for 2 hours at 25°C and 63 hours at 5°C the specimens were washed 3 x 20 min in 0.05 M phosphate buffer pH 7. Dehydration was carried out using a series of ethanol washings (50%, 70%, 80% and 96%) followed by 3 washes of 99% ethanol (30 min for each ethanol concentration). After additional treatment with 2 x 2 hrs in petroleum (Shellsol™ D70k, Q7712) and 2 x 2 hrs in paraffin with 7% 20 beeswax, the samples were embedded in paraffin. Cross-sections of 12.5 µm were made on a Supercut 2050 Reichart Jung pyramitome.

Immunology

25 Tissue sections were pre-incubated with 20% swine serum in TBS (0.5 M Tris/HCl pH 7.6, 0.15 M NaCl, 0.1% Triton X-100) for 30 min before treatment for 1 hour with PME antibodies were diluted 1: 50 in TBS. Excess antibody was removed by washing with TBS for 5 x 5 min. After washing the sections were incubated for 30 min with secondary antibodies coupled with alkaline phosphatase 1:20 in TBS buffer. 30 Surplus of secondary antibody was removed by TBS washing as described above. Before staining the sections were treated with veronal acetate buffer pH 9.2 for 5 min and then stained with Fast Red and Naphtol AS-BI phosphate (Sigma no N4875) for

20 min. Excess reagent was removed by washing with water. Controls were run in parallel and treated with pre-immune serum.

Results

5

Immunological localizations with the antibody raised against peel PME showed that PME is situated in large amounts in the outer cell layer of the lamella between the segments, in the core and in the outer cell layer of the juice sacs and also in the inner cell layer of the albedo (see Figure 1). These results demonstrate that the antibody
10 against peel PME cross reacts with PME from the flesh (the flesh consists of segments, see Figure 1) indicating a high homology between the PME located in the peel and in the flesh, respectively.

STUDY 2

15

PME from orange peel was purified in large amounts. In this regard, approximately 70 mg PME was isolated from 5 kg orange peels. The purified PME was then used in the application test using milk protein - i.e. a drinking yoghurt. In this test, the block-wise enzymatically de-esterified pectin improved protein stabilising properties
20 compared to non-de-esterified pectin, presumably because of the formed block structure. The final product also had a favourable viscosity.

Enzyme Isoforms

25

Whilst not wishing to be bound by theory, it is believed that the PME of the present invention can exist in at least two isoforms. Isoform S has a molecular weight of about 36 kD, and can be referred to as the "short PME". Isoform L has a molecular weight of about 64 kD, and can be referred to as the "long PME".

30

It is also believed that the Isoform L is more heat stable than Isoform S. Also, it is believed that Isoform S starts the initial de-esterification step in a block-wise manner and is then superseded by Isoform L.

In other words, it is believed that Isoform L may have a greater affinity to partially de-esterified pectin than Isoform S.

It is believed that the heat stability of Isoform L may be due to the amino acid sequence represented as SEQ.I.D.No. 5.

Further studies have shown that the gene for Isoform L has an N-terminal extension upstream of the signal sequence. This is shown diagrammatically in Figure 12.

ISOLATION AND CHARACTERIZATION OF cDNAs ENCODING PECTIN METHYL ESTERASE OBTAINABLE FROM ORANGE

MATERIALS AND METHODS FOR MOLECULAR BIOLOGY

1. MATERIALS

Oranges (*Citrus sinensis*) var. Navel originating from Morocco were used.

2. DNA

Genomic DNA was isolated as described by Dellaporta S.L.*et al* (1983) Plant Mol Biol Rep 1(4):19-21. Plasmid DNA was isolated as described in EP-B-0470145.

3. RNA

Total RNA was isolated from mature orange fruits. The outer portion of the flesh and the inner portion of the albedo layer (see Figure 1) of a Navel orange fruit were used for the RNA isolation following the procedure described by Logemann J., Schell J. and Willmitzer L. (1987) Anal. Biochem 163:16-20. "Improved Method for the isolation of RNA from Plant Tissues".

4. PCR

5 Total orange RNA was used to make reverse PCR with the rTth Reverse PCR Kit (Perkin Elmer) according to the suppliers instructions with the following temperature cycling:

Reverse transcription:

10 70°C 2 min
60°C 2 min
50°C 2 min
45°C 5 min
40°C 5 min
30°C 10 min
15 42°C 10 min
70°C 2,5 min
5°C soak

Amplification (PCR):

20 94°C 2 min
92°C 1 min
45°C 2 min
72°C 2 min in 40 cycles
25 72°C 5 min
5°C soak

5. CLONING OF PCR FRAGMENTS

30 PCR fragments were cloned into the *EcoRV* site of the vector pT7Blue (Novagen) following the suppliers instructions.

6. DNA SEQUENCING

Double stranded DNA was sequenced essentially according to the dideoxy method of Sanger *et al.* (1979) using the Auto Read Sequencing Kit (Pharmacia) and the Pharmacia LKB A.L.F. DNA sequencer (Ref: Sanger, F., Nicklen, S., and Coulson, A. R. (1979). DNA sequencing with chain-determinating inhibitors. Proc. Nat. Acad. Sci. USA 74: 5463-5467). The primers used for sequencing are listed below (presented 5' to 3'):

10 UNI (M13-20 Primer) - 17 Mer
GTAAACGACGGCCAGT

REV - 19 MER
GGAAACAGCTATGACCATG

15 01 - 20 MER
GACAACGGCAACGAGCCTCA

02 - 22 MER
20 GCACTTGTAATAACCCTAAAT

03 - 20 MER
CAGGGTAGTTTCCCGACGTA

25 04 - 20 MER
TACGTCGGGAACTACCCTG

05 - 21 MER
CTCCTGGAAGCTTCATTGCTG

30 06 - 20 MER
GAAGCTTCTTGAAGAGAACG

07 - 20 MER

CGTTCTCTTCAAGAAGAAGCTTC

08 - 22MER

5 GAGGATAGCATGATGAGGCTCG

09 - 22 MER

GCAGTTCACAAAGAACTGGCGG

10 010 - 22 MER

CCTCATGATCATCATGTCAGTG

011 - 21 MER

GCTCCTCAGGGAGGCACTAAG

15

012 - 21 MER

CAGCAATGAAGCTTCCAGGAG

013 - 21 MER

20 CTTAGTGCCTCCCTGAGGAGC

014 - 18 MER

GCCACCGCCTGGTGCTTT

25 015 - 18 MER

AAAGCACCAGGCGGTGGC

016 - 20 MER

GCGGGATGCGTTGTCAGACG

30

017 - 20 MER

GAGGCACTAAGCGGTATATT

O18 - 18 MER

GCAACTGTAGCAGACTTG

O19 - 20 MER

5 CACTGACATGATGATCATGA

O20 - 20 MER

CAATTAAGCAGTTCACAAAG

10 O21 - 20 MER

AATATACCGCTTAGTGCCTC

15 The sequenced nucleotide sequence is shown as SEQ.I.D. No. 3 (derived from pO17) and SEQ.I.D.No. 4 (derived from pO34). The N-terminal sequence is shown as SEQ.I.D.No. 6.

7. SCREENING OF THE LIBRARY

20 A cDNA library in *lambda zapII* (Stratagene) which had been prepared from mRNA isolated the flesh and albedo layer of the orange fruit was screened with the appropriate radio labelled PCR probe. The screening was performed according to the suppliers instructions except that the pre-hybridization and hybridization was performed in 2 x SSC, 0.1% SDS, 10 x Denhardt's and 100 μ g/ml denatured salmon sperm DNA. Hybridization was overnight at 67°C. The filters were washed twice
25 in 2 x SSC, 0.1% SDS, twice in 1 x SSC, 0.1% SDS and twice in 0.1 x SSC, 0.1% SDS.

8. PROBE

30 The cloned PCR fragment was isolated from the pT7 blue vector by digestion with the appropriate restriction enzymes. The fragment was separated from the vector by agarose gel electrophoresis and the fragment purified and radiolabelled using the Ready to Go™ DNA labelling kit (Pharmacia).

9. SOUTHERN ANALYSIS

Genomic orange DNA or plasmid DNA were digested with appropriate restriction enzymes, transferred to HybondN⁺™ membranes and hybridized following the
5 suppliers (Amersham) instructions.

10. *IN SITU* HYBRIDIZATION EXPERIMENTS

In situ hybridization technique is based upon the principle of hybridization of an
10 antisense ribonucleotide sequence to the mRNA. The technique is used to visualize areas in microscopic sections where said mRNA is present. In this particular case the technique was used to localize the mRNA encoding the enzyme pectin methyl esterase in sections of *C. sinensis*.

15 Preparation of tissue samples for *in situ* hybridization.

Thin slices of the middle part of a mature orange fruit were fixed by fixation with FAA fixation (45% ethanol, 5% formalin (40% paraformaldehyde) and 5% acetic acid) and incubation for 2 h at 25°C and 63 h at 5°C. The samples were washed for
20 3 x 20 min in a 0.05 M phosphate buffer, pH 7. Dehydration was carried out using a series of ethanol washes (50%, 70%, 80%, 96%) ending with 3 washes in 99% ethanol. Each wash was for 30 min. The samples were then treated with petroleum (Shellsol™ D70k, Q7712) in 2 x 2 h and for another 2 x 2 h in paraffin with 7% beeswax. After this the samples were embedded in paraffin. Cross-sections of
25 12,5µm were made using a Supercut 2050 Reichart Jung pyramitome.

Preparation of ³⁵S labelled probes for *in situ* hybridization

A 501 bp PCR fragment from a second PCR amplification was cloned into the
30 pT7blue vector (Novagen) in both directions. The pT7 vector contains a T7 promoter. The transcription of the antisense RNA and the sense RNA were driven by the SP7 promoter after digesting the plasmids with BamHI. A Maxiscript Kit™

(Ambion) was used with the following modifications. The transcripts were run on a 6% sequencing gel to remove the incorporated nucleotide and eluted with the elution buffer supplied with the T7 RNA polymerase *in vitro* transcription kit (Ambion). The transcripts contained 55 non-coding nucleotides at one end and also 9 non-coding nucleotides at the other end. For hybridization 10^7 cpm/ml of the ^{35}S labelled probe was used.

In situ hybridization was performed essentially as described by Langedale J.A.(1994) in the Maize Handbook-M. Freeling and V. Walbot, eds. pp 165-180 Springer-Verlag, New York, Inc. The hybridization temperature was found to be optimal at 57°C . After washing at 57°C the sections were covered with Kodak K-5 photographic emulsion and left for 3 days at 5°C in the dark.

11. RESULTS

The following sequence information was used to generate primers for the PCR reactions mentioned below and to check the amino acid sequence generated by the respective nucleotide sequences - see attached SEQ.I.D.No.s 7-19.

Peptide sequences of pectin methyl esterase used for generation of primers

Asn Cys Asp Met Leu Ala Tyr Gln Asp Thr Leu Tyr (PE492B)

Val ILe Thr Ser Ala Thr Glu Ala Gln Ala Phe Thr Pro Gly Ser Phe Ile Ala Gly Ser Ser Trp Leu Gly Ser Thr Gly Phe (PE701)

The amino acid sequence (PE492B,4-7) used to generate primer A (Met Leu Ala Tyr Gln Asp Thr)

Primer A

ATG(CT)T(GATC)GC(GATC)TA(TC)CA(AG)GA(TC)AC 256 mix

The amino acid sequence (PE701,7-13) used to generate primer B (Glu Ala Gln Ala Phe Thr Pro)

Primer B

5

GT(AG)AA(GATC)GC(TC)TG(GATC)GC(TC)TC 128 mix
(the sequence corresponds to the complementary strand)

Generation of PCR DNA fragment partially encoding pectin methyl esterase

10

The amino acid sequences of the two non-overlapping peptides (described above) were used to generate mixed oligonucleotides. These were used as primers for reverse transcription and the following amplification (PCR) of total RNA. The resulting PCR clone was sequenced and had an insert of 501 bp. The deduced amino acid sequence from the nucleotide sequence corresponded nearly perfectly with the peptide sequences given in SEQ.I.D.No.s 7-19.

15

In situ hybridization analysis

20

The *in situ* hybridization experiments (see Materials and Methods) with the riboprobes (produced from the appropriate PCR clones) against the mRNA of pectin methyl esterase, showed strong hybridization in the outer cell layer of the lamella between the segments (see Figure 1). Strong signals were also obtained from the outer cell layer of the juice sacs, the inner cell layer of the albedo and the core. These results correspond very well with the immunological localizations results seen with the antibody raised against peel PME as described previously.

25

Southern analysis

30

Southern analysis showed that additional copies of the isolated PME genes (represented by the cDNA clones) are present in the *C. sinensis* genome. These other PME genes were rather homologous with the ones represented in pO17 and pO34.

In this regard, we observed in each lane one band of a strong hybridization signal, two bands of a middle hybridization signal and at least two more bands of a weaker signal compared with the rest. This pattern indicates that *C. sinensis* has at least between 5 and 7 copies of PME genes in the genome.

5

Isolation and characterization of novel PME cDNAs

A cDNA library in *lambda zapII* (Stratagene) which had been prepared as described in Materials and Methods was screened with the radiolabelled 501 bp insert from the PCR clone. Several hybridizing clones were identified and plasmid DNA was excised *in vivo* according to the suppliers instructions. The size of the cDNA inserts in the clones were determined by digestion with EcoRV and SmaI followed by agarose gel electrophoresis. One clone pO34 had an insert size of approximately 2 kb whereas another clone pO17 had an insert size of approximately 1.4 kb. These clones were selected for further analysis. The nucleotide sequences were determined and are shown as SEQ.I.D. NO. 3 and SEQ.I.D. No. 4 for pO17 and pO34, respectively.

The open reading frame in O34 starting at nucleotide 29 and ending at nucleotide 1780 encodes a PME of 584 amino acids including a putative signal peptide. A possible cleavage site between glycine at position 46 and isoleucine at position 47 can be predicted according to the rules of von Heijne, G.(1986) Nucl Acids Res 14, 4683-4690 "A new method for predicting signal sequence cleavage sites", thereby giving a long mature PME enzyme of 538 amino acids having a calculated molecular weight of 58386 dalton. The molecular weight of the long PME including the signal sequence can be calculated to 63502 dalton. pO34 nucleotide sequence includes an untranslated 5' region of 29 nucleotides and a untranslated 3' region of 186 nucleotides, ending in a poly A tail.

The open reading frame in O17 starts at nucleotide 18 and terminates at nucleotide 1103. It encodes a short PME of 362 amino acids including a signal peptide of 44 amino acids. A putative cleavage site can be predicted between glutamine at position 44 and the serine at position 45 leaving a mature short PME starting with the amino

acid sequence Ser-Ser-Ser-Val-Thr-Pro. This N-terminal amino acid sequence is identical to the N-terminal amino acid sequencing of the purified short type of PME as described in the biochemical section. The amino acids sequences obtained from the purified enzyme were aligned with the deduced mature amino acid sequence of pO17. Full identity was found for the peptide fragments.

There was nearly full identity between all the sequenced peptides and the deduced amino acid sequence in pO17 and also with the amino acid sequence deduced from pO34, the long PME. In this regard, pO17 differs at one amino acid position (no. 24 in the sequence encoding the mature polypeptide). The mature short PME protein has a calculated molecular weight of 33954 dalton. The calculated molecular weight of the short PME form including the signal peptide is 39088 dalton. O17 includes a 5' untranslated region of 17 nucleotides and a 3' untranslated region of 180 nucleotides, ending in a polyA tail.

The major difference between the long and the short PME enzyme is an N-terminal extension of 220 amino acids present in the mature enzyme derived from O34 and presented as SEQ.I.D.No. 5. The corresponding nucleotide sequence encoding this region of the long PME enzyme is shown as SEQ.I.D.No. 6.

EXPRESSION OF PME IN MICRO-ORGANISMS

The DNA sequence encoding either the long or the short form of orange PME was introduced into micro-organisms to produce a recombinant enzyme in large quantities having a high specific activity to be used for enzymatic treatment of pectin.

EXPRESSION IN *PICCHIA PASTORIS*

Construction of pJK10, pJK11 and pJK12 (see Figs. 13-15)

pO34 plasmid DNA was used as template DNA in a PCR reaction with the following set of primers:

5'-GAATTCATTGTCGCCGGAGTGAAC-3' with an *EcoRI* site at one end and

5'-AAGACCAGAGACCTATGGATCCAC-3' with a *BamHI* site near the end.

5 Combining AmpliTaq^R DNA Polymerase (Perkin Elmer), template DNA, dATP, dGTP, dCTP and dTTP and the two primers in the following buffer:

60 mM Tris-HCl (pH 8.5), 15 mM (NH₄)₂SO₄ and 1.5 mM MgCl₂ and using the following temperature cycle:

10

94°C 2 min

94°C 1 min

55°C 2 min

15

72°C 2 min in 35 cycles

72°C in 7 min

5°C soak

20

produced the expected PCR product of 1690 bp which was purified and subcloned into the vector pT7Blue (from Novagen) following the suppliers instructions.

25

Resulting clones (called pT7-O34) containing an *EcoRI-BamHI* fragment of the expected size were further verified by DNA sequencing (see Materials and Methods for Molecular Biology). A pT7-O34 subclone containing the correct sequence was digested with *EcoRI* and *BamHI* and the fragment of 1685 bp was purified and subcloned into the *Pichia pastoris* vector pHIL-S1 (from Invitrogen) digested with the same enzymes.

30

The sequence encoding the mature long form of PME was cloned in this way in frame with the PHO1 secretion signal (S) in the vector. The resulting plasmid is

called pJK10 and is shown in figure 13.

To create pJK11 (see figure 14) the *EcoRI-BamHI* fragment of the pT7-O34 clone was further subcloned into a pBSK- vector (from Stratagene) digested with the same enzymes. Then, an *EcoRI-NorI* fragment from one of the resulting clones (verified by DNA sequencing) was subcloned into the *Pichia pastoris* expressions vector pPIC9 (from Invitrogen) digested with the same enzymes. This places the reading frame encoding the mature protein, of the long form of PME, downstream and in frame with the alpha-factor secretion signal (S) in pPIC9, see figure 14.

In addition, the *EcoRI-NorI* fragment from pT7-O34 was also subcloned into the pPIC9K (Invitrogen) vector creating the pJK12 plasmid shown in figure 15. The only difference between pJK11 and pJK12 is a gene encoding resistance for kanamycin and this is situated in pJK12.

Both the PHO1 secretion signal in pJK10 and the alpha secretion signal in pJK11 and pJK12 can direct the secretion of the mature polypeptide encoding the long form of PME.

Construction of pJK20, pJK21 and pJK22

pO17 plasmid DNA was used as a template DNA in a PCR reaction with the following primers:

5'-GAATTCTCCTCGTCGGTGACACCG-3' with an *EcoRI* site at one end and

5'-AAGACCAGAGACCTATGGATCCAC-3' with a *BamHI* site near the end.

The template DNA, AmpliTaq^R polymerase, dATP, dGTP, dCTP and dTTP were combined with the buffer (60 mM Tris-HCl, pH 8.5; 15 mM (NH₄)₂SO₄ and 1.5 mM MgCl₂) and placed in a thermoblok with the following temperature cycle:

94°C 2 min

94°C 1 min

55°C 2 min

5

72°C 2 min in 35 cycles

72°C in 7 min

5°C soak

10

This produced an expected PCR band of 1008 bp which was purified and subcloned into pT7Blue (Novagen). The resulting clones were verified by DNA sequencing as explained above. A plasmid with the correct sequence was digested with *EcoRI* and *BamHI* and the resulting fragment further subcloned into the pHIL-S1 vector (Invitrogen) the resulting plasmid is called pJK20 (shown in figure 16) and in this, is the region encoding the mature polypeptide of the short form of PME situated in frame and downstream of the PHO1 secretion signal.

15

Plasmid pJK21 and pJK22 were constructed in the same manner as explained for the pJK11 and pJK12 except that the *EcoRI*-*NotI* fragment was obtained from the pBSK-O17 clone. Figure 17 and 18 show the resulting plasmids where the region encoding the mature polypeptide is situated downstream, and in frame, of the alpha secretion signal in respectively pPIC9 and pPIC9K.

20

25 Introduction of the long or the short form of PME into *Pichia pastoris* by spheroplast formation and transformation

The plasmids pJK10, pJK11, pJK12, pJK20, pJK21 and pJK22 were introduced in separate experiments into *Pichia pastoris* GS115 cells.

30

In this regard, spheroplasts were prepared from the GS115 cells growing in yeast extract peptone dextrose medium (YPD) at 28-30°C. The preparation of spheroplasts

and the transformation procedure were performed as described in the *Pichia* expression kit: Instruction Manual (Invitrogen). The resulting *Pichia pastoris* Mut⁺ or Mut^s transformants were then analyzed for the expression of the recombinant PME gene by assaying the supernatant for PME activity using the methods described in:
5 Materials and Methods for Biochemistry.

Screening of transformants for high expression of the long or the short form of PME

10 Putative positive transformants were further grown in minimal medium (either Minimal Dextrose Medium or Minimal Glycerol Medium as specified in the *Pichia* expression kits Instruction Manual, Invitrogen) and the genomic DNA was isolated and analysed by PCR as described in the Instruction Manual.

15 Positive clones found in the initial screen, which also produced a PCR band of the expected size, were selected and further grown in a flask at 28-30°C following the procedure described in the *Pichia* Instruction Manual.

20 At least 10 verified recombinant clones of each construct (pJK10, pJK11, pJK12, pJK20, pJK21 and pJK21) were screened for secretion of PME and their relative expression level were measured over time by sampling every 2 to 6 hours. The cells in the samples were pelleted and the supernatant assayed for PME activity following the methods explained in: Materials and Methods for Biochemistry.

25 The clones obtained after transformation with either pJK12 or pJK22 were preselected using the integrated Kanamycin resistance gene as the selector gene as described by Scorer C.A. *et al.* (1994) Bio/Technology vol 12, pp181-184 and Laroche Y. *et al* (1994) Bio/Technology vol 12, pp 1119-1124. In this way multiple copy integration transformants were obtained and further screened as described above.

30 Transformants representing either the long or the short form of PME and shown to express the recombinant protein in high levels were selected and further analyzed by Western Blot analysis (see Materials and Methods for Biochemistry).

Purification and characterization of recombinant PME

Recombinant PME protein was purified from the cultures supernatant using the procedures described in the Materials and Methods for Biochemistry as necessary.

5

The presumed high temperature stability of the long form of PME was verified by testing the enzyme activity after incubation at different temperatures as explained in the section: Characterization and kinetic data. The purified recombinant enzymes (both the long and the short PME form) were further characterized as explained in the: Characterization and kinetic data section. These analyses showed that both types had a pH optimum around 7-8 and the temperature stability of the short recombinant PME was found to be between 10 and 40°C and up to 80°C for the long recombinant PME.

10

EXPRESSION IN *ASPERGILLUS NIGER*

15

In another embodiment, pO34 or pO17 was digested with the appropriate restriction enzymes and the coding sequence for the long or the short form of PME of the present invention was cloned into the *Aspergillus* expression vector pBAMTE1 (containing methyl tryptophan resistance promoter from *Neurospora crassa*) for expression in *Aspergillus niger* (Pall *et al.* (1993) Fungal Genet Newslett. vol 40 pp 59-62).

20

The protoplasts were prepared according to Daboussi *et al.* (Curr Genet (1989) vol 15. pp453-456) using lysing enzymes Sigma L-2773 and the lyticase Sigma L-8012. Transformation of the protoplasts followed the protocol stated by Buxton *et al.* (Gene (1985) vol 37 pp 207-214) except that for plating the transformed protoplasts the protocol laid out in Punt *et al.* (Methods in Enzymology (1992) vol 216 pp447-457) was followed but with the use of 0.6% osmotic stabilised top agarose.

25

30

The results showed that purified orange PME activity was obtainable from *Aspergillus niger* cultures.

USES

EFFECT OF ORANGE PME TREATED PECTIN ON THE VISCOSITY AND STABILITY OF PROTEIN DRINKS

5

METHODS

Enzymatic treatment of pectin with PME derivable from orange

10 A batch of enzymatically treated pectin was prepared as follows:

125 g pectin was dissolved in hot water under efficient stirring. 45.3 g NaCl (reagent grade) was added and the volume adjusted to 4.0 l with water. This solution was stirred until the salt had dissolved. The pectin solution was cooled to 40°C and the
15 pH was increased to pH 7.0, using 1 N NaOH (reagent grade) and efficient stirring. An appropriate sample of orange PME was added and the enzymatic reaction continued until the desired degree of estrification was achieved. The pH was kept constant at pH 7 by automatic dosage of 1 N NaOH (reagent grade) during the incubation period, and the enzymatic reaction was followed by the consumption of
20 NaOH.

When the pectin sample had reached the desired degree of de-esterification the NaOH addition was stopped, the pH of the solution lowered to about 3.0 by addition of 2 % HCl. The pectin solution was then heated to 70°C for 5 min to completely
25 inactivate the enzyme. The treated pectin was precipitated with 1 volume of isopropanol, washed with 60% isopropanol and pressed to about 50 % dry matter. The enzymated pectin batch was then air dried at 40°C and finally milled to a dry powder.

PROTOCOLDetermination of pectin samples for calcium sensitivity index (CF)

5 Calcium sensitivity is measured as the viscosity of a pectin dissolved in a solution with 57.6 mg calcium/g pectin divided by the viscosity of exactly the same amount of pectin in solution, but without added calcium. A non calcium sensitive pectin has a CF value of 1.

10 4.2 g pectin sample is dissolved in 550 ml hot water with efficient stirring. The solution is cooled to about 20°C and the pH adjusted to 1.5 with 1N HCl. The pectin solution is adjusted to 700 ml with water and stirred. 145 g of this solution is measured individually into 4 viscosity glasses. 10 ml water is added to two of the glasses (double determinations) and 10 ml of a 250 mM CaCl₂ solution is added to the other two glasses under stirring.

15 50 ml of an acetate buffer (0.5 M, pH about 4.6) is added to all four viscosity glasses under efficient magnetic stirring, thereby bringing the pH of the pectin solution up over pH 4.0. The magnets are removed and the glasses left overnight at 20°C. The viscosities are measured the next day with a Brookfield viscometer. The calcium sensitivity index is calculated as follows:

20

$$\text{CF} = \frac{\text{Viscosity of a solution with 57.6 mg Ca}^{2+} / \text{g pectin}}{\text{Viscosity of a solution with 0.0 mg Ca}^{2+} / \text{g pectin}}$$

25

Determination of pectin samples degree of esterification (%DE)

To 50 ml of a 60 % isopropanol and a 5 % HCl solution is added 2.5 g pectin sample and stirred for 10 min. The pectin solution is filtered through a glass filter and washed with 15 ml 60 % isopropanol/5 % HCl solution 6 times followed by further washes with 60% isopropanol until the filtrate is free of chlorides. The filtrate is dried overnight at 80°C.

20.0 ml 0.5 N NaOH and 20.0 ml 0.5 N HCl is combined in a conical flask and 2 drops of phenolphthalein is added. This is titrated with 0.1 N NaOH until a permanent colour change is obtained. The 0.5 N HCl should be slightly stronger than the 0.5N NaOH. The added volume of 0.1 N NaOH is noted as V_0 .

0.5 g of the dried pectin sample (the filtrate) is measured into a conical flask and the sample is moistened with 96% ethanol. 100 ml of recently boiled and cooled distilled water is added and the resulting solution stirred until the pectin is completely dissolved. Then 5 drops of phenolphthalein are added and the solution titrated with 0.1 N NaOH (until a change in colour and pH is 8.5). The amount of 0.1 N NaOH used here is noted as V_1 . 20.0 ml of 0.5 N NaOH is added and the flask shaken vigorously, and then allowed to stand for 15 min. 20.0 ml of 0.5 N HCl is added and the flask is shaken until the pink colour disappears. 3 drops of phenolphthalein are then added and then the resultant solution is titrated with 0.1 N NaOH. The volume 0.1 N NaOH used is noted as V_2 .

The degree of esterification (% DE: % of total carboxy groups) is calculated as follows:

$$\% \text{ DE} = \frac{V_2 - V_0}{V_1 + (V_2 - V_0)}$$

Yoghurt production

Standardised skimmed milk (prepared by mixing powdered milk with an adequate volume of water) was heated at 90°C in 5 min and then homogenized at 200 kp/cm² and the milk cooled to 31°C. Yoghurt culture was added and the milk fermented to about pH 4.0. The yoghurt was cooled to about 20°C and the pectin sample was added as a saturated sugar solution (about 65% sugar) and stirred for 15 min. The pH was adjusted to pH 4.0 with lactic acid. The yoghurt was pasteurized at 88°C for 15 seconds and homogenized at 150 kp/cm², then cooled to 20°C and filled into sterile 250 ml blue cap bottles (200 ml/bottle).

The composition of the final product was: 7.6 % MSNF (milk solid content), 9.15 % sugar and 0.25 % or 0.35 % pectin sample giving total solids of 17.0 % or 17.10 %, respectively.

Viscosity determination of yoghurt drink

The viscosity of a yoghurt sample was determined (double determinations) using either a Bohlin Rheometer™ (supplied by Bohlin Instruments) with shear rates 18.5-46.0 or a using a StressTech™ (Rheologica instruments AB) with the same shear rates.

Protein stability measured by a centrifugation test

20 g of a sample (e.g. drinking yoghurt) is centrifuged at 10°C, 2300xg for 20 min. The supernatant is discarded and the centrifuge glass placed upside down for 30 min. The glass is weighed and the % sedimentation was calculated as follows:

$$\% \text{ Sedimentation} = \frac{\text{Wgt of glass after centri} - \text{Wgt of glass}}{\text{Wgt of sample}} \times 100$$

Where Wgt= weight and centri= centrifugation

Protein stability as judged by particle size distribution in the sample

Particle size distribution of a yoghurt sample was determined by the use of a Malvern 2600 Easy™ sizer. The particle size is determined by laser light scattering by this method. 1 ml yoghurt sample was added to 9 ml de-aerated buffer solution (30.7 % 0.1 M citric acid, 19.3 % 0.2 M Na₂HPO₄ and 50.0 % water) and mixed. The de-aerated buffer is added to the measuring glass and the mixture of sample/buffer is added droplet by droplet until the optimum concentration is obtained. The average particle size is calculated from the measurements.

A yoghurt with the average particle size below about 3 µm is considered rather stabile while a yoghurt with an average particle size over about 10 µm and higher is considered not to be stabile for long term storage.

Determination of long-term stability

Samples were stored at 4°C or at ambient temperature and the whey separation was measured (in mm of whey at the top of the sample, in the bottle). The sample was filled in 250 ml blue cap bottles (double determinations). The depth of the sample in each case was approximately 70 mm - which corresponded to the 200 ml mark for each bottle.

EXAMPLE 1

SOUR MILK DRINK

The purpose of adding pectin to a sour milk drink (e.g. a yoghurt drink) is to produce a drink that remains physically homogenous during the bacteriological and organoleptic shelf life of the drink. In addition the treatment of the yoghurt drink for long term storage destabilizes the protein in the drink giving rise to a drink with a sandy mouthfeel and showing rather quickly syneresis, if pectin is not added.

Treatment of the pectin

5 A commercially available high ester pectin; Grindsted™ Pectin URS (Ultra Rapid Set pectin type) was chosen as the mother pectin, due to its high ester level (% DE of 82, see figure 19). The treatment with the orange PME enzyme of this mother pectin is explained in the method section.

10 The enzymatic reaction was stopped and the resulting experimental pectin (called pectin no 1944-96-2) was investigated for its degree of esterification, by the method described in the method section. In addition, to compare the two pectin types, the mother pectin and the treated pectin, with a known good commercial drinking yoghurt pectin type, the Grindsted™ Pectin AM453 was included in the experiments.

15 The relative calcium sensitivity of the three chosen pectins was determined as explained in the method section: Determination of pectin samples calcium sensitivity index (CF), and the results are shown in the Table below.

Pectin-type	Δ CF	DE %
Grindsted™ Pectin URS	1.1	82
Pectin 1944-96-2	1.4	76
Grindsted™ Pectin AM453	> 20	72

20 De-esterification of the Grindsted™ Pectin URS mother pectin from 82 % DE down to 76 % DE gave nearly no change in the calcium sensitivity of the these two pectins (a Δ CF of 1 have no sensitivity). A pectin with measurable calcium sensitivity could be produced by further treatment with the orange PME enzyme on the mother pectin down to 70% DE, since this pectin had a Δ CF of 14.

Results of Yoghurt/Drinking Yoghurt Analysis

Yoghurt was produced as explained in the method section. The three pectins (Grindsted™ Pectin URS, Pectin 1944-96-2 and Grindsted™ Pectin AM453) were used individually in the following recipes:

7.6 % MSNF (milk solid content), 9.15 % sugar and 0.25 % or 0.35% pectin sample giving total solids of 17.0 % or 17.10 %, respectively in the final product.

The quality of the individual yoghurt produced was investigated by their sedimentation % seen in the centrifugation test, by measuring the particle size in the yoghurt, by measuring the viscosity and by examination of possible whey separation during long time storage, as described in the method section.

The sedimentation of the yoghurt produced with the three pectin types are presented in the Table below.

SEDIMENT (IN %) OF THE YOGHURT

Pectin-type	Concentration - 0.25%	Concentration - 0.35%
Grindsted™ Pectin URS	29.40	21.02
Pectin 1944-96-2	1.53	2.95
Grindsted™ Pectin AM453	2.20	1.85

The results are the average of from one to four individual production.

It is clear that the mother pectin (URS type) had a high sedimentation % and consequently the yoghurt produced showed whey separation and was unstable at both pectin concentrations used (0.25 and 0.35 %). This is not surprising since normally the URS pectin type cannot be used for stabilization of heat treated yoghurt types for long time storage.

The yoghurt produced with the Pectin 1944-96-2 showed stability at both pectin dosages used and low sedimentation as seen by the above results, and no whey separation after over 75 days of storage. In comparison, the excellent Grindsted™ Pectin AM453 normally used in yoghurt production also showed low sedimentation, as expected, and produced stabile yoghurts with no whey separation (see results above).

By treating the mother URS pectin with orange PME an unsuitable pectin was made suitable as a stabilizing agent in the yoghurt, and this treated pectin behave just as well as an excellent commercial stabilizer.

Further examination of the produced yoghurts by particle size determination (see the methods section) were performed and the results are shown in the Table below.

PARTICLE SIZE (IN μ M) OF THE YOGHURT

Pectin-type	Concentration - 0.25%	Concentration - 0.35%
Grindsted™ Pectin URS	9.32	6.41
Pectin 1944-96-2	1.33	1.55
Grindsted™ Pectin AM453	1.40	1.53

The average particle size (the number corresponding to the D(4.3)) fraction using the Malvern instrument, is shown) of the yoghurt produced with the mother URS pectin is high, as expected. Again the results are an average of one to four productions.

- 5 The average particle size of the yoghurt produced from both the pectin 1944-96-2 and the Grindsted™ Pectin AM453 are small at both pectin dosages used (see results above). Again showing that the yoghurt produced with these pectin types are suitable to produce stabile yoghurts.
- 10 Finally, and very importantly, in the case of drinking yoghurt production for long time storage, the viscosity was determined and the results are shown in the Table below.

VISCOSITY (IN MPa s) OF THE YOGHURT

15

20

Concentration / Pectin- type	Concentration - 0.25 %	Concentration - 0.35 %
Grindsted™ Pectin URS	55	40
Pectin 1944-96-2	12	18
Grindsted™ Pectin AM453	24	43

- 25 Since the mother URS pectin could not stabilize the yoghurt the viscosity obtained is, as expected rather high at both pectin concentrations. The excellent Grindsted™ Pectin AM453 which produced stabile yoghurts with low sedimentation and low particle size showed a viscosity of about half the viscosity seen with the Grindsted™ Pectin URS pectin at the 0.25 % pectin dosage and approximately the same as the URS pectin at the 0.35 % pectin dosage - irrespective of the fact that only the AM453
- 30 pectin produced a stabile yoghurt.

In both the URS and the AM453 cases the higher viscosity seen could partly be attributed to the amount of pectin added, especially this seems to be the situation in the AM453 pectin case since an increase in the amount of pectin added (from 0.25 to 0.35 %) produced nearly twice as viscous a yoghurt.

5

The viscosity obtained with the orange treated PME pectin 1944-96-2 shows a dramatic fall in viscosity compared with the mother URS pectin at 0.25% concentration. This is partly due to the stabilization of the yoghurt with the 1944-96-2 pectin but not the only reason since the AM453 yoghurt has twice as high a viscosity at that pectin dosage. Indeed, adding 0.35 % of the 1944-96-2 pectin to the yoghurt only increases the viscosity with 6 units (compared with the 0.25 % dosage) while the viscosity increases with 19 units going from 0.25 % to 0.35 % in the AM453 case.

10

The orange PME treated pectin 1944-96-2 can stabilize yoghurt having a very low viscosity, despite the pectin dosage was 0.25% (or 0.35 %), which gave nearly twice as high a viscosity using other untreated pectins - e.g. Grindsted™ Pectin AM453.

15

This is a new and very important development for the production of sour milk drinks.

20

By treating the Grindsted™ Pectin URS with orange PME a new pectin type is created which in contrast to the mother pectin can stabilize yoghurt and most importantly shows much lower viscosity than normally used pectins. Other high ester pectin can also be improved by treatment with the orange PME.

25

EXAMPLE 2

Whey Juice Drink

The modified pectin according to the present invention (as prepared above) was used in a whey juice drink as follows:

30

5

Sweet or acid whey	42.00%
Fruit juice	40.00%
Sugar	8.00%
Sodium citrate	0.20%
PME modified pectin	0.20%
Grindsted Flavouring	+
Water	9.60%

10

Dry PME modified pectin, sodium citrate and sugar were mixed and then dissolved in water at 80°C. This pectin solution was cooled to below 5°C and whey was added at 5°C. Grindsted Flavouring (supplied by Danisco Ingredients, Danisco A/S) and juice were added slowly and the pH in the sample mixture was adjusted with citric or lactic acid to pH 4.0. The sample mixture was aged for approx. 30 min under agitation. The pasteurisation was performed at 80°C/15 seconds and homogenisation at 200 bar (2900 psi). The samples were cooled to 20°C and filled aseptically in containers.

15

20

Sample tests were analysed after 24 hours, 1 months and 6 months incubation at room temperature. The investigation analysis included viscosity measurements, stability index particle size and long-term stability - the protocols for which are described above.

25

The results showed improved stability and a long-term stability in the whey juice drink processed with PME modified pectin compared to the reference pectin employed in the control tests. In addition, the whey juice drink had a favourable viscosity which was lower than the control drinks.

30

The whey juice drink was also processed with plant PME modified lime and lemon pectin, respectively, - i.e. modified with the PME of the present invention. The results demonstrate that PME modified pectin has improved protein stability compared to the non-modified pectin and also compared to the reference pectin.

EXAMPLE 3**MILK/FRUIT JUICE DRINK**

5 Grindsted™ URS (obtained from Danisco Ingredients, Danisco A/S) was modified with the PME as described for the yoghurt drink. The modified pectin was used in a milk/fruit juice drink which contained:

10	Skimmed milk	45.00%
	Fruit juice	40.00%
	Sugar	5.00%
	PME modified pectin	0.25%
	Grindsted Flavouring	+
	Water	9.75%

15 Dry PME modified pectin and sugar were mixed and then dissolved in water at 80°C. The pectin solution was cooled to below 5°C and milk was added at 5°C. Grindsted Flavouring and juice were added slowly and the pH in the sample mixture was adjusted (if necessary) with citric or lactic acid to pH 4.0. The sample mixture was aged, pasteurised and homogenised as described for whey juice drink. The samples
20 were cooled to 20°C and filled aseptically in containers.

The results showed improved stability - including long-term stability - in the milk/fruit juice drink processed with PME modified pectin compared to the reference pectin employed in the control tests. In addition, the milk/fruit juice drink had a
25 favourable viscosity which was lower than the control drinks. Improved functionality was also observed.

The milk/fruit juice drink can also be processed with lime and lemon pectin modified with the PME of the present invention.

30

EXAMPLE 4**Whey stability**

5 The enzymatic modified pectins were tested at pH 4.0. The pH of the resultant pectin solutions were adjusted to pH 4.0 with KOH/HCl. The pectin concentration was adjusted to 1.0%. The pectins were tested in concentrations of 0.1% - 0.25 %. Pectin, Jenness Buffer (see below) and whey solution (see below) were mixed and heated at 96°C for 25 min. After cooling to room temperature the absorbance is measured at 500 nm.

10

Dry Blend Jenness Buffer:

Dry Powder Jenness (described in Jenness, R and Koops, J *Preparation and Properties of a salt solution which simulates milk ultrafiltrate*, Nederlands Melk- en
15 Zuiveltijdschrift, vol 16 nr 3, pp 153-164, 1962):

15.80 g KH_2PO_4
5.08 g K_3 citrate
17.91 g Na_3 citrate, 2. H_2O
20 1.80 g K_2SO_4
13.20 g CaCl_2 , 2. H_2O
5.02 g Mg_3 citrate, H_2O
3.00 g K_2CO_3
10.78 g KCl

25

Buffer solution:

Aqueous solution of 7.5900 g/l Dry Powder Jenness with a pH of 4.0.

Whey Solution

A concentrate of whey protein is freeze dried and pulverized. A solution of 0.40% w/w whey protein is prepared in Jenness Buffer at pH 4.0.

5

Pectin solutions

1% w/w aqueous solutions of pectins are made with a pH of 4.0.

10

Mixture concentrations:

Pectin, Jenness Buffer and Whey solution are mixed as indicated in the Table below. The mixtures are heated at 96°C for 25 minutes and after cooling to room temperature the samples are measured on a spectrophotometer at 500 nm.

15

μl pectin solution	μl Jenness Buffer	μl whey solution	μl total volume
500	2000	2500	5000
750	1750	2500	5000
1000	1500	2500	5000
1250	1250	2500	5000

20

Results

25

The results are shown in the Table below. The quoted absorbance value at 500 nm is a mean value taken from two measurements. For comparison between the different types of pectin and the enzymatic modified pectins according to the present invention the index for non-modified pectin Grindsted™ Pectin 3450 (supplied by Danisco Ingredients, Danisco A/S) is set to 100.

30

An Index of > 100 indicates poorer protein stability than using the sample 3450. An Index of 100 indicates similar stability to the sample 3450. An Index of < 100 indicates better protein stability than using the sample 3450. An Index of 95 or < 95 indicates very good protein stability.

5

10

Pectin type	0.10% pectin	0.15% pectin	0.20% pectin	0.25% pectin
Grindsted™ pectin 3450	100	100	100	100
Grindsted™ pectin URS	127	140	155	161
5 min enz.	100	108	115	113
10 min enz.	98	106	111	111
15 min enz.	92	94	100	100
20 min enz.	90	95	100	99

15

As can be seen from the results the Grindsted™ pectin URS pectin modified according to the present invention exhibits favourable properties and in some instances very good properties for increasing stability when compared to the reference Grindsted™ pectin 3450 and the un-modified Grindsted™ pectin URS pectin.

20

EXAMPLE 5

Laban Drink with long Shelf life, low pH

25

The Laban drink is an acidified milk drink with a pH value below 4.2. The laban drink consists of Laban base mixed with pectin solution. The formulation for laban base is:

Anhydrous milk fat	2.8%
Skimmed milk powder	10.0%
Grindsted flavouring	+
Water	87.2%

5

Standardised whole milk (anhydrous milk fat and skimmed milk powder) is homogenised at 75-80°C and pressure of 200 bar (2900 psi) followed by pasteurisation at 90-95°C for 5-10 min. After culturing to pH around 4.0 the pH is
10 adjusted to 3.8 - 4.2 with citric or lactic acid. Pectin solution is then added. The mixture is then agitated until it is a homogeneous mixture. It is then pasteurised at 90-95°C for 10-15 seconds and further homogenised at 150-200 bar. After cooling to 20-25°C the product is filled aseptically in containers.

15 After a few days, non-stabilised laban drink often show syneresis.

However, addition of the enzymatically modified pectin according to the present invention prevents syneresis and improves the viscosity.

20 In addition, the product has a pronounced yoghurt taste and a long shelf life.

EXAMPLE 6

Orange Juice (Protein-enriched)

25 Orange juice drink is an acidified drink (pH approx. 4) containing 2 % DANPROLACT 40™ (Central Soya, Aarhus A/S), 6% sugar, 10% orange concentrate, 0.4% lemon concentrate, 0.2% pectin and 81.4% water. The product is an orange juice drink enriched with soya protein. The product is pasteurised and homogenised. After cooling to 20-25°C the product is filled aseptically in
30 containers and can be stored for approx. 6 months at room temperature. Addition of enzymatically modified pectins according to the present invention in orange drink

(protein-enriched) showed favourable properties - such as long term stability - and it had a good mouth feel.

EXAMPLE 7

5 Antibody production

Antibodies were raised against the enzyme of the present invention by injecting rabbits with the purified enzyme and isolating the immunoglobulins from antiserum according to procedures described according to N Harboe and A Ingild
10 ("Immunization, Isolation of Immunoglobulins, Estimation of Antibody Titre" In A Manual of Quantitative Immunoelectrophoresis, Methods and Applications, N H Axelsen, *et al* (eds.), Universitetsforlaget, Oslo, 1973) and by T G Cooper ("The Tools of Biochemistry", John Wiley & Sons, New York, 1977).

15 Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the present invention.

In the following pages a number of sequence listings are presented which have been consecutively numbered from SEQ.I.D. No. 1 - SEQ.I.D. No. 19, which represent
20 nucleotide sequences and amino acid sequences.

SEQUENCES

SEQ.I.D. NO. 1

5	MIKNMTDTDM	MIMRTSNNRK	LIEETSTVDG	WPAWLSTGDR	RLQSSSVTP	50
	NVVVAADGSG	NFKTVAAAVA	AAPOGGTKRY	IIRIKAGVYR	ENVEVTKKHK	100
	NIMFIGDGRT	RTIITGSRNV	VDGSTTFKSA	TVAVVGEGL	ARDITFQNTA	150
	GPSKHQAV	RVGADLSAFY	NCDMLAYQDT	LYVHSNRQFF	VNCLIAGTVD	200
	FIFGNAAVL	QNCDIHARKP	NSGQKNMVT	QGRADPNQNT	GIVIQSRIG	250
10	ATSDLKPVG	SFPTYLGRPW	KEYSRTVIMQ	SSITDVIHPA	GWHEWDGNFA	300
	LNTLFYGEHQ	NAGAGAGTSG	RVKWKGRVI	TSATEAQFT	PGSFIAGSSW	350
	LGSTGFPSL	GL				362

SEQ.I.D. NO. 2

15	MTRIKEFFTK	LSESSTNQNI	SNIPKKKKKL	FLALFATLLV	VAAVIGIVAG	50
	VNSRKNSGDN	GNEPHHAILK	SSCSSTRYPD	LCFSAIAAVP	EASKVTSQK	100
	DVIEMSLNIT	TTAVEHNYFG	IQKLLKRTNL	TKREKVALHD	CLETIDETLD	150
	ELHKALEDLE	EYPNKKLSQ	HADDLKTLM	AAMTNQGTCL	DGFSHDDANK	200
20	HVRDALSDGQ	VHVEKMCSNA	LAMIKNMTDT	DMIMRTSNN	RKLIEETSTV	250
	DGWPWLSTG	DRLLQSSSV	TPNVVVAADG	SGNFKTVAAS	VAAAPQGGTK	300
	RYIIRIKAGV	YRENVEVTKK	HKNIMFIGDG	RRTIITGSR	NVVDGSTTFK	350
	SATVAVVGE	FLARDITFQ	TAGPSKHQAV	ALRVGADLSA	FYNCDMLAYQ	400
	DTLYVHSNRQ	FFVNCLIAGT	VDFIFGNAAA	VLQNCDIHAR	KPNSGQKNMV	450
25	TAQGRADPNQ	NTGIVIQSR	IGATSDLKPV	QGSFPTYLGR	PWKEYSRTVI	500
	MQSSITDVIH	PAGWHEWDGN	FALNTLFYGE	HQONAGAGAGT	SGRVKWKGR	550
	VITSATEAQA	FTPGSFIAGS	SWLGSTGFPF	SLGL		584

SEQ.I.D. NO. 3

30	GTAGCAATGC	GCTTGCTATG	ATCAAGAACA	TGACTGACAC	TGACATGATG	50
	ATCATGAGGA	CTTCAAACAA	CAGGAAGCTG	ATAGAGGAGA	CCAGTACGGT	100
	TGATGGGTGG	CCGGCGTGGC	TGTCCACCGG	AGACAGGAGG	CTGTTGCAGT	150
	CCTCGTCGGT	GACACCGAAC	GTGGTGGTGG	CAGCAGATGG	CAGCGGAAAC	200
35	TTTAAGACGG	TGGCGGCAGC	GGTGGCGGCG	GCTCCTCAGG	GAGGCACTAA	250
	GCGGTATATT	ATTAGGATTA	AAGCCGGTGT	TTATCGGGAA	AATGTTGAGG	300
	TGACAAAGAA	GCATAAAAT	ATAATGTTCA	TGGGTGACGG	GAGGACTAGA	350
	ACTATCATCA	CAGGAAGTAG	AAATGTGGTT	GATGGAAGCA	CAACTTTCAA	400
	GTCTGCTACA	GTTGCTGTTG	TTGGTGAAGG	ATTCTTGGCC	CGAGACATTA	450
40	CATTCCAAAA	CACAGCCGGC	CCCTCAAAGC	ACCAGGCGGT	GGCACTACGA	500
	GTGGGAGCTG	ACCTTTCAGC	ATTTTACAAT	TGCGATATGT	TAGCTTACCA	550
	AGACACACTC	TACGTCCACT	CGAACCGCCA	GTTCTTGTG	AACTGCTTAA	600
	TTGCTGGCAC	GTTTGATTTT	ATTTTGGTA	ACGCTGCAGC	CGTGTACAA	650
	AATTGTGACA	TCCATGCACG	AAAGCCCAAT	TCCGGCCAAA	AAAATATGGT	700
45	CACAGCCCAA	GGCAGGGCTG	ACCCTAACCA	AAACACCGGC	ATTGTCATTC	750

	AAAAATCTAG GATTGGTGCC ACCTCCGATT TAAAACCGGT TCAGGGTAGT	800
	TTCCCCGACGT ACCTCGGCAG GCCCTGGAAG GAGTACTCGA GGACGGTGAT	850
	CATGCAGTCA TCGATTACTG ACGTGATCCA CCCTGCCGGG TGGCACGAGT	900
	GGGATGGTAA CTTGCGGTTG AACACATTGT TTTACGGAGA GCATCAGAAC	950
5	GCCCGAGCCG GTGCCGGAAC TTCAGGGAGA GTGAAATGGA AGGGATTTAG	1000
	GGTTATTACA AGTGCTACCG AGGCTCAAGC TTTTACTCCT GGAAGCTTCA	1050
	TTGCTGGTAG TAGCTGGCTG GGCTCCACTG GTTTCCCAT TCTCCCTTGGT	1100
	TTGTAATATT CACTAGGAGT TTTAATTAAT ATGTTTTGTA TTAGTGGATC	1150
	CATAGGTCTC TGGTCTTTCA ATTTGTAATA TTTGATTGAG CGTGTCTTAT	1200
10	TCGTGGCTTC GATTTCAACA ATACTATTGT GTGATTAACA AGAAATAAAA	1250
	TAGCATGGGA AGAATAATAA TTTCCGGCTT CTTTAAAAAA AAAAAAAAAA	1300
	AAAAAAAAAA AAAAAAAAAA AAA	1323
	SEQ.I.D. NO. 4	
15	CTTTTGTTCT CTCTTATCGA GAAAAAAAT GACCCGCATA AAAGAATTCT	50
	TCACAAAAC TTTCTGAATCT TCTACCAACC AAAACATTTT CAATATTCCC	100
	AAGAAAAAA AGAAACTATT CTTAGCTCTT TTTGCAACGC TACTCGTTGT	150
	CGCTGCCGTA ATCGGCATTG TCGCCGGAGT GAACTCAAGA AAAAATCCG	200
20	GCGACAACGG CAACGAGCCT CATCATGCTA TCCTCAAATC ATCATGTAGC	250
	AGCACAAAGT ACCCGGACTT ATGCTTTTTCG GCTATTGCTG CCGTTCCAGA	300
	GGCCTCCAAA AAGGTGACAA GCCAAAAGGA CGTTATTGAG ATGTCCTTAA	350
	ACATCACAAC AACAGCCGTG GAACACAAC TCTTCGGGAT TCAGAAGCTC	400
	TTGAAGAGAA CGAATCTCAC CAAACGGGAA AAGGTTGCTC TCCATGACTG	450
25	TCTTGAGACG ATCGATGAGA CTCTTGATGA GTTACACAAA GCCGTCGAGG	500
	ATCTTGAGGA GTACCCGAAC AAGAAATCTT TATCACAGCA TGCGGATGAT	550
	CTCAAAACCC TAATGAGTGC CGCGATGACC AATCAGGGGA CGTGTCTTGA	600
	TGGGTTCTCT CATGATGATG CTAATAAGCA CGTGCGGGAT GCGTTGTCAG	650
	ACGGCCAGGT TCATGTTGAG AAGATGTGTA GCAATGCGCT TGCTATGATC	700
30	AAGAACATGA CTGACACTGA CATGATGATC ATGAGGACTT CAAACAACAG	750
	GAAGCTGATA GAGGAGACCA GTACGGTTGA TGGGTGGCCG GCGTGGCTGT	800
	CCACCGGAGA CAGGAGGCTG TTGCAGTCCT CGTCGGTGAC ACCGAACGTG	850
	GTGGTGGCAG CAGATGGCAG CGGAAACTTT AAGACGGTGG CGGCATCGGT	900
	GGCGGCGGCT CCTCAGGGAG GCACTAAGCG GTATATTATT AGGATTAAAG	950
35	CCGGTGTTTA TCGGGAAAAT GTTGAGGTGA CAAAGAAGCA TAAAAATATA	1000
	ATGTTTCATCG GTGACGGGAG GACTAGAACT ATCATCACAG GGAGTAGAAA	1050
	TGTGGTTGAT GGAAGCACAA CTTTCAAGTC TGCTACAGTT GCTGTTGTTG	1100
	GTGAAGGATT CTTGGCCCGA GACATTACAT TCCAAAACAC AGCCGGCCCC	1150
	TCAAAGCACC AGGCGGTGGC ACTACGAGTG GGAGCTGACC TTTCAGCATT	1200
40	TTACAATTGC GATATGTTAG CTTACCAAGA CACACTCTAC GTCCACTCGA	1250
	ACCGCCAGTT CTTTGTGAAC TGCTTAATTG CTGGCACGGT TGATTTTATT	1300
	TTTGGAACG CTGCAGCCGT GTTACAAAAT TGTGACATCC ATGCACGAAA	1350
	GCCCAATTCC GGCCAAAAAA ATATGGTCAC AGCCCAAGGC AGGGCTGACC	1400
	CTAACCAAAA CACCGGCATT GTCATTCAAA AATCTAGGAT TGGTGCCACC	1450
45	TCCGATTTAA AACCGGTTCA GGGTAGTTTC CCGACGTACC TCGGCAGGCC	1500

	CTGGAAGGAG	TACTCGAGGA	CGGTGATCAT	GCAGTCATCG	ATTACTGACG	1550
	TGATCCACCC	TGCCGGGTGG	CACGAGTGGG	ATGGTAACTT	CGCGTTGAAC	1600
	ACATTGTTTT	ACGGAGAGCA	TCAGAACGCC	GGAGCCGGTG	CCGGAAC TTC	1650
	AGGGAGAGTT	AAATGGAAGG	GATTTAGGGT	TATTACAAGT	GCTACCGAGG	1700
5	CTCAAGCTTT	TACTCCTGGA	AGCTTCATTG	CTGGTAGTAG	CTGGCTGGGC	1750
	TCCACTGGTT	TCCCATTCTC	CCTTGGTTTG	TAATATTCAC	TAGGAGTTTT	1800
	AATTAATATG	TTTTGTATTA	GTGGATCCAT	AGGTCTCTGG	TCTTTCAATT	1850
	TGTAATATTT	GATTGAGCGT	GTCTTATTCG	TGGCTTCGAT	TTCACAAATA	1900
	CTATTGTGTG	ATTAACAAGA	AATAAAATAG	CATGGGAAGA	ATAATAATTT	1950
10	CCGGCTTCTT	TAAATTAAAA	AAAAA			1975

SEQ.I.D. NO. 5

	IVAGVNSRKN	SGDNGNEPHH	AILKSSCSST	RYPDLCFSAI	AAVPEASKKV	50
15	TSQKDVIEWS	LNITTTAVEH	NYFGIQKLLK	RTNLTREKV	ALHDCLETID	100
	ETLDELHKAV	EDLEEYPNKK	SLSQHADDLK	TLMSAAMTNQ	GTCLDGFSDH	150
	DANKHVRDAL	SDGQVHVEKM	CSNALAMIKN	MTDMDMMIMR	TSNNRKLIEE	200
	TSTVDGWPAW	LSTGDRRLQ				220

20 SEQ.I.D. NO. 6

	ATTGTCGCCG	GAGTGAAC TC	AAGAAAAAAC	TCCGGCGACA	ACGGCAACGA	50
	GCCTCATCAT	GCTATCCTCA	AATCATCATG	TAGCAGCACA	AGGTACCCGG	100
	ACTTATGCTT	TTCCGGCTATT	GCTGCCGTTC	CAGAGGCCTC	CAAAAAGGTG	150
25	ACAAGCCAAA	AGGACGTTAT	TGAGATGTCC	TTAAACATCA	CAACAACAGC	200
	CGTGGAACAC	AACTACTTCG	GGATTCAGAA	GCTCTTGAAG	AGAACGAATC	250
	TCACCAAACG	GGAAAAGGTT	GCTCTCCATG	ACTGTCTTGA	GACGATCGAT	300
	GAGACTCTTG	ATGAGTTACA	CAAAGCCGTC	GAGGATCTTG	AGGAGTACCC	350
	GAACAAGAAA	TCTTTATCAC	AGCATGCGGA	TGATCTCAA	ACCCTAATGA	400
30	GTGCCGCGAT	GACCAATCAG	GGGACGTGTC	TTGATGGGTT	CTCTCATGAT	450
	GATGCTAATA	AGCACGTGCG	GGATGCGTTG	TCAGACGGCC	AGGTTCATGT	500
	TGAGAAGATG	TGTAGCAATG	CGCTTGCTAT	GATCAAGAAC	ATGACTGACA	550
	CTGACATGAT	GATCATGAGG	ACTTCAAACA	ACAGGAAGCT	GATAGAGGAG	600
	ACCAGTACGG	TTGATGGGTG	GCCGGCGTGG	CTGTCCACCG	GAGACAGGAG	650
35	GCTGTTGCAG					660

SEQ.I.D. NO. 7

40	PE511 (14 aa)	Ser-ala-thr-val-ala-val-val-gly-glu-gly-phe-leu-ala-arg
----	---------------	---

SEQ.I.D. NO. 8

45	PE3252 (4 aa)	Tyr-ile-ile-arg
----	---------------	-----------------

SEQ.I.D. NO. 9

PE8 (8 aa) Asn-ile-met-phe-ile-gly-asp-gly

5 SEQ.I.D. NO. 10

PE21 (21 aa) Ile-gly-ala-thr-ser-asp-leu-lys-pro-val-gln-gly-ser-
phe-pro-thr-tyr-leu-gly-arg-pro

10 SEQ.I.D. NO. 11

PE492D (15 aa) xxx-ser-ala-thr-val-ala-val-val-gly-glu-gly-phe-leu-
ala-arg

15 SEQ.I.D. NO. 12

PE492C (11 aa) xxx-ala-tyr-pro-gly-gln-ile-thr-ser-asn-met

20 SEQ.I.D. NO. 13

PE492B (12 aa) Asn-cys-asp-met-leu-ala-tyr-gln-asp-thr-leu-tyr

SEQ.I.D. NO. 14

25 PE492A (16 aa) Val-ile-thr-ser-ala-thr-glu-ala-gln-ala-phe-thr-pro-
gly-ser-phe

SEQ.I.D. NO. 15

30 PE701 (28 aa) Val-ile-thr-ser-ala-thr-glu-ala-gln-ala-phe-thr-pro-
gly-ser-phe-ile-ala-gly-ser-ser-trp-leu-gly-ser-thr-
gly-phe

SEQ.I.D. NO. 16

35 PE594 (13 aa) Ile-ala-gly-ser-ser-trp-leu-gly-ser-thr-gly-phe-pro

SEQ.I.D. NO. 17

40 PE7 (19 aa) Asn-met-val-thr-ala-gln-gly-arg-ala-asp-pro-asn-gln-
asn-thr-gly-ile-val-ile

SEQ.I.D. NO. 18

5 PE 251 (38 aa) xxx-arg-ile-gly-ala-thr-ser-asp-leu-lys-pro-val-gln-
gly-ser-phe-pro-thr-tyr-leu-gly-arg-pro-(trp)-lys-
glu-tyr-(ser)-arg-(thr)-val-ile-met-gln-ser-ser-ile-
thr

SEQ.I.D. NO. 19

10 PE 201 (27 aa) xxx-xxx-ile-gly-ala-thr-ser-asp-leu-lys-pro-val-gln-
gly-ser-phe-pro-thr-tyr-leu-gly-arg-pro-xxx-lys-glu-
tyr

15 SEQ.I.D. NO. 20

PE 22 (21 aa) Ser-arg-ile-gly-ala-thr-ser-asp-leu-lys-pro-val-gln-
gly-ser-phe-pro-thr-tyr-leu-gly


20 aa 2 (arg) could be replaced with val
aa 3 (ile) could be replaced with met
aa 6 (thr) could be replaced with val

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>30</u> , lines <u>22 to 30</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution The National Collections of Industrial and Marine Bacteria Limited (NCIMB)	
Address of depositary institution (including postal code and country) 23 St. Machar Drive Aberdeen Scotland AB2 1RY United Kingdom	
Date of deposit 6 July 1995, 12 July 1995*	Accession Number * NCIMB 40749; NCIMB 40750
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

<p>For receiving Office use only</p> <p><input checked="" type="checkbox"/> This sheet was received with the international application</p> <p>Authorized officer</p> <p style="text-align: center;">  C.A.J.A. PASCHE </p>	<p>For International Bureau use only</p> <p><input type="checkbox"/> This sheet was received by the International Bureau on:</p> <p>Authorized officer</p>
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RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli K12 SOLR-p034	NCIMB 40749
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by:	
<input type="checkbox"/> a scientific description	
<input checked="" type="checkbox"/> a proposed taxonomic designation	
(Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 6 July 1995 (date of the original deposit) ¹	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Address:	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 11 July 1995
NCIMB Ltd 23 St Machar Drive Aberdeen Scotland UK AB2 1RY	Terence Dudo

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

Danisco Biotechnology,
Langebrogade 1,
PO Box 17,
DK-1001 Copenhagen K,
Denmark.

INTERNATIONAL FORM

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY STATEMENT
IS ISSUED

I DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Address: AS ABOVE	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40749 Date of the deposit or of the transfer: 6 July 1995
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 7 July 1995 ² . On that date, the said microorganism was <div data-bbox="105 1312 267 1375"> <input checked="" type="checkbox"/> ³ viable </div> <div data-bbox="105 1396 406 1459"> <input type="checkbox"/> ³ no longer viable </div>	

- ¹ Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
- ² In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.
- ³ Mark with a cross the applicable box.

V. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED⁴

V. INTERNATIONAL DEPOSITARY AUTHORITY

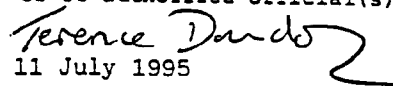
Name:

NCIMB Ltd

Address:

23 St Machar Drive
Aberdeen Scotland
UK AB2 1RYSignature(s) of person(s) having the power
to represent the International Depositary
Authority or of authorized official(s):

Date: 11 July 1995



⁴ Fill in if the information has been requested and if the results of the test were negative.

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RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli K12 SOLR - p017	NCIMB 40750
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by:	
<input type="checkbox"/> a scientific description	
<input checked="" type="checkbox"/> a proposed taxonomic designation	
(Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 12 July 1995 (date of the original deposit) ¹	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name:	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):
Address:	Date:
NCIMB Ltd 23 St Machar Drive Aberdeen Scotland UK AB2 1RY	21 July 1995 Terence Dando

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

—
Danisco Biotechnology
Langebrogade 1
P O Box 17
DK-1001 Copenhagen K
Denmark

INTERNATIONAL FORM

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

—
NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY STATEMENT
IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: As above Address:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40750 Date of the deposit or of the transfer: 12 July 1995
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 19 July 1995 ² . On that date, the said microorganism was <div style="display: flex; flex-direction: column; align-items: flex-start;"> <div style="margin-bottom: 10px;"> <input checked="checked" type="checkbox"/> ³ viable </div> <div> <input type="checkbox"/> ³ no longer viable </div> </div>	

¹ Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

I. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED⁴

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name:

NCIMB Ltd

Address:

23 St Machar Drive
Aberdeen Scotland
UK AB2 1RYSignature(s) of person(s) having the power
to represent the International Depositary
Authority or of authorized official(s):

Date:

Terence Dando
21 July 1995⁴ Fill in if the information has been requested and if the results of the test were negative.

CLAIMS

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- 5 1. A process comprising adding to an acidic environment, which contains at least one protein, a block-wise enzymatically de-esterified pectin, wherein the pectin is a high ester pectin.
2. A process according to claim 1 wherein the block-wise enzymatically de-esterified pectin is prepared by recombinant DNA techniques.
- 10 3. A process according to claim 1 or claim 2, wherein the acidic environment is an aqueous solution, preferably wherein the aqueous solution is a beverage.
4. A process according to claim 3, wherein the beverage is a drinking yoghurt, a fruit juice or a beverage comprising whey protein.
- 15 5. A process according to any one of the preceding claims, wherein the protein is derived from or is derivable from or is in a dairy product, such as milk or cheese.
6. A process according to claim 5, wherein the protein is casein or whey protein.
- 20 7. A process according to any one of the preceding claims, wherein the acidic environment has a pH of from about 3.5 to about 5.5, preferably wherein the acidic environment has a pH of from 4 to about 5.5.
- 25 8. A process according to any one of the preceding claims, wherein the acidic environment has a pH of about 4.
9. A process according to any one of the preceding claims wherein the block-wise enzymatically de-esterified pectin prepared by recombinant DNA techniques contains from about 70% to about 80% ester groups, preferably about 76% ester groups.
- 30

10. A process according to any one of the preceding claims, wherein the block-wise enzymatically de-esterified pectin prepared by recombinant DNA techniques is insensitive to Ca^{2+} ions according to the Protocol as mentioned in the Examples.
- 5 11. A process according to any one of the preceding claims, wherein the block-wise enzymatically de-esterified pectin prepared by recombinant DNA techniques has a high molecular weight.
- 10 12. A process according to any one of the preceding claims, wherein the block-wise enzymatically de-esterified pectin prepared by recombinant DNA techniques is prepared by treating a pectin with a recombinant pectin methyl esterase that de-esterifies two or more adjacent galacturonic acid residues of the pectin on at least substantially all of the pectin chains.
- 15 13. A process according to any one of the preceding claims, wherein the recombinant pectin methyl esterase is derived from a PME obtainable from a plant.
14. A process according to claim 13, wherein the plant is a fruit.
- 20 15. A process according to claim 14, wherein the fruit is a citrus fruit.
16. A process according to claim 15, wherein the citrus fruit is an orange.
- 25 17. A process according to any one of claims 13 to 16, wherein the recombinant pectin methyl esterase is derived from a PME obtainable from the lamella or albedo of an orange.
- 30 18. A process according to any one of the preceding claims, wherein the block-wise enzymatically de-esterified pectin is prepared by treating a pectin with a recombinant enzyme comprising any one of the amino acid sequences shown as SEQ.I.D. No.1 or SEQ.I.D. No. 2, or a variant, derivative or homologue thereof, including combinations thereof.

19. A process according to any one of the preceding claims, wherein the block-wise enzymatically degraded pectin is prepared by treating a pectin with an recombinant enzyme that is obtainable by expression of the PME coding sequence contained in NCIMB 40749 or NCIMB 40750, or a variant, derivative or homologue thereof, or combinations thereof; or by expression of a nucleotide sequence comprising the nucleotide sequence shown as SEQ.I.D. No. 3 or SEQ.I.D. No. 4, or a variant, derivative or homologue thereof, or combinations thereof.
20. A process according to any one of claims 12 to 19 wherein the block-wise enzymatically de-esterified pectin is prepared by treating the pectin with the recombinant pectin methyl esterase in the presence of sodium ions.
21. A process according to claim 20 wherein the block-wise enzymatically de-esterified pectin is prepared by treating the pectin with the recombinant pectin methyl esterase in the presence of NaCl, NaNO₃ or Na₂SO₄.
22. A method of block-wise enzymatically de-esterifying a pectin comprising treating the pectin with a recombinant enzyme comprising any one of the amino acid sequences shown as SEQ.I.D. No.1 or SEQ.I.D. No. 2, or a variant, derivative or homologue thereof, including combinations thereof.
23. A method according claim 22, wherein the block-wise enzymatically degraded pectin is prepared by treating a pectin with a recombinant enzyme that is obtainable by expression of the PME coding sequence contained in NCIMB 40749 or NCIMB 40750, or a variant, derivative or homologue thereof.
24. A method according to claims 22 to 23 wherein the block-wise enzymatically de-esterified pectin is prepared by treating the pectin with the recombinant pectin methyl esterase in the presence of sodium ions.

25. A method according to claim 24 wherein the block-wise enzymatically de-esterified pectin is prepared by treating the pectin with the recombinant pectin methyl esterase in the presence of NaCl, NaNO₃ or Na₂SO₄.

5 26. A recombinant enzyme comprising any one of the amino acid sequences shown as SEQ.I.D. No.1 or SEQ.I.D. No. 2, or a variant, derivative or homologue thereof, including combinations thereof.

10 27. A nucleotide sequence coding for the recombinant enzyme according to claim 26, wherein the nucleotide sequence comprises any one of the sequences shown as SEQ. I.D. No. 3 or SEQ. I.D. No.4, or a variant, derivative or homologue thereof.

15 28. A nucleotide sequence coding for the recombinant enzyme according to claim 26 wherein the nucleotide sequence is obtainable from NCIMB 40749 or NCIMB 40750, or a variant, derivative or homologue thereof. or a variant, derivative or homologue thereof.

20 29. A construct expressing or comprising the recombinant enzyme according to claim 26 or a nucleotide sequence according to claim 27 or claim 28.

30. A vector expressing or comprising a construct according to claim 29 or the recombinant enzyme according to claim 26 or a nucleotide sequence according to claim 27 or claim 28.

25 31. A combination of constructs comprising at least a first construct expressing or comprising the recombinant enzyme according to claim 26 or a nucleotide sequence according to claim 27 or claim 28; and a second construct comprising a gene of interest (GOI) and a promoter.

32. A cell, tissue or organ expressing or comprising a vector according to claim 30 or a construct according to claim 29 or the recombinant enzyme according to claim 26 or a nucleotide sequence according to claim 27 or claim 28 or a combination of constructs according to claim 32.

5

33. A transgenic organism expressing or comprising the invention according to any one of claims 26 to 32.

34. NCIMB 40749 or NCIMB 40750.

10

35. A recombinant PME enzyme which is immunologically reactive with an antibody raised against a purified recombinant enzyme according to claim 26, but not a tomato PME enzyme.

36. Use of the recombinant enzyme according to claim 26 or claim 35 to reduce the number of ester groups in a pectin.

15

37. Use of the recombinant enzyme according to claim 26 or claim 35 to de-esterify pectin in a block-wise manner.

20

38. Use of the recombinant enzyme according to claim 26 or claim 35 to affect the calcium sensitivity of pectin.

39. Use of the recombinant enzyme according to claim 26 or claim 35 to esterify pectin containing free carboxyl groups.

25

40. A pectin obtained by use of the recombinant enzyme according to claim 26 or claim 35.

41. Use of a pectin according to claim 40 to prepare a foodstuff.

30

90

42. Use of a pectin according to claim 40 to stabilise a protein in an acidic environment without adversely affecting the viscosity of the environment.
- 5 43. A combination of enzymes comprising a recombinant enzyme according to claim 26 or claim 35 and a fungal PME or other pectin degrading enzyme.
- 10 44. An amino acid sequence comprising the sequence shown as SEQ.I.D. No. 5, or a variant, derivative or homologue thereof, suitable for use in imparting or increasing heat stability to a protein.
45. A nucleotide sequence comprising the sequence shown as SEQ.I.D. No. 6, or a variant, derivative or homologue thereof, suitable for use in expressing an amino acid sequence for imparting or increasing heat stability to a protein.
- 15 46. A recombinant nucleotide sequence coding for the enzyme according to claim 26.
47. A recombinant PME enzyme substantially as described herein.
- 20 48. A pectin prepared by use of a recombinant PME enzyme substantially as described herein.

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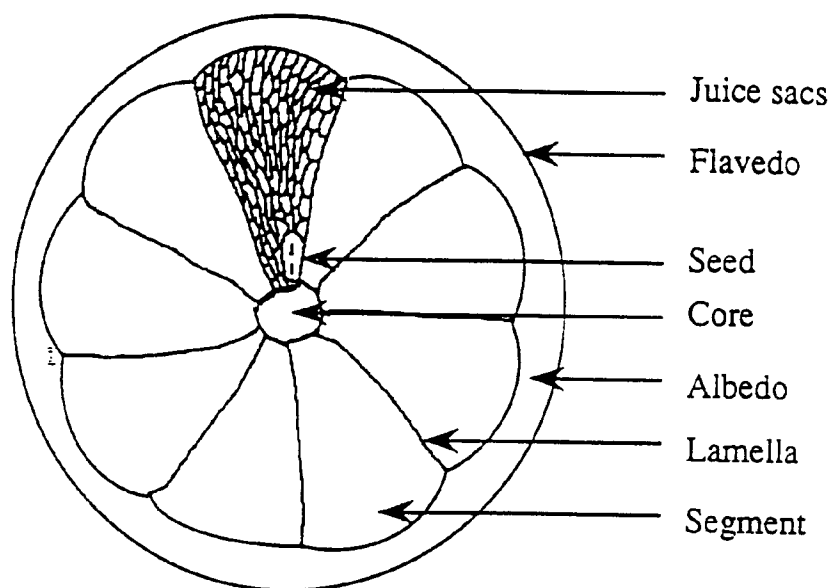
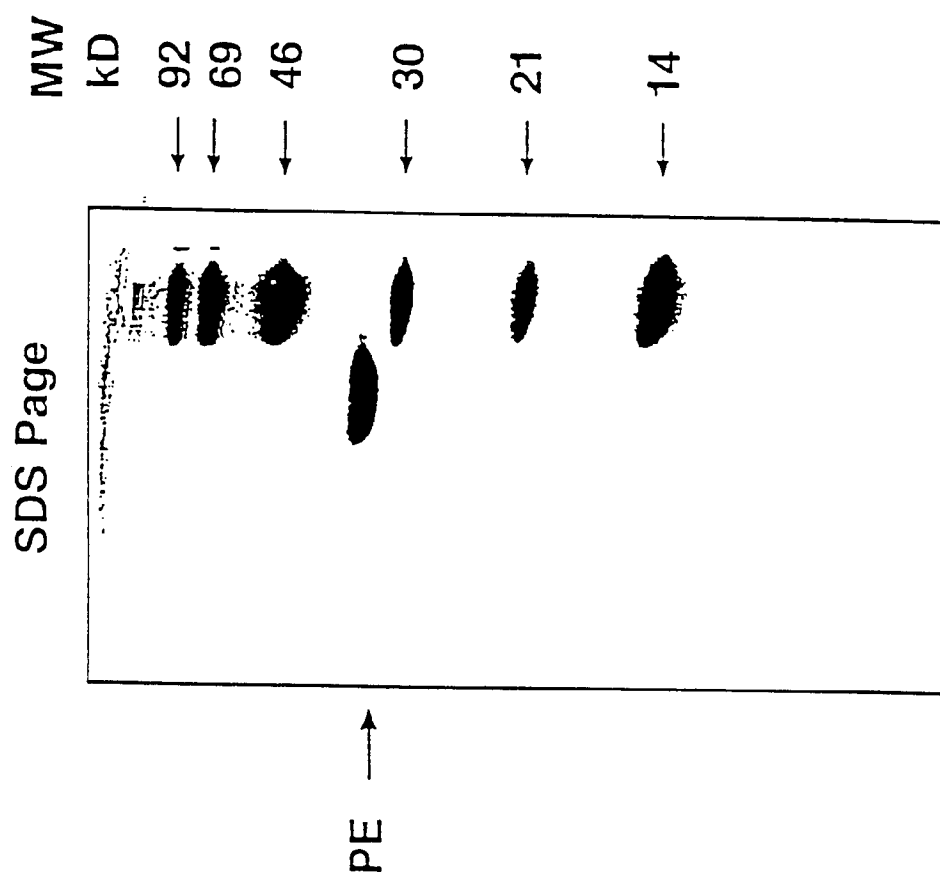


Figure 1. Schematic diagram of a citrus fruit

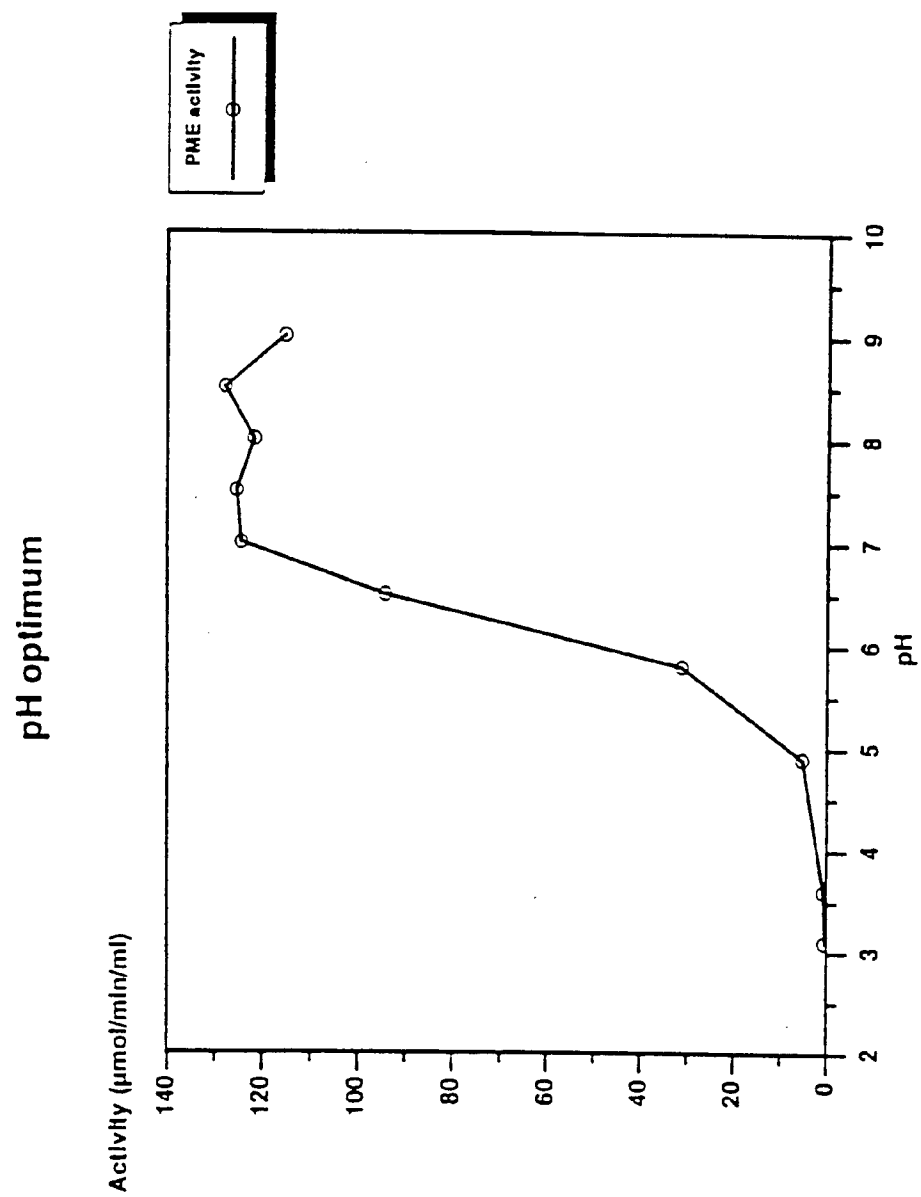
2/18

Fig. 2



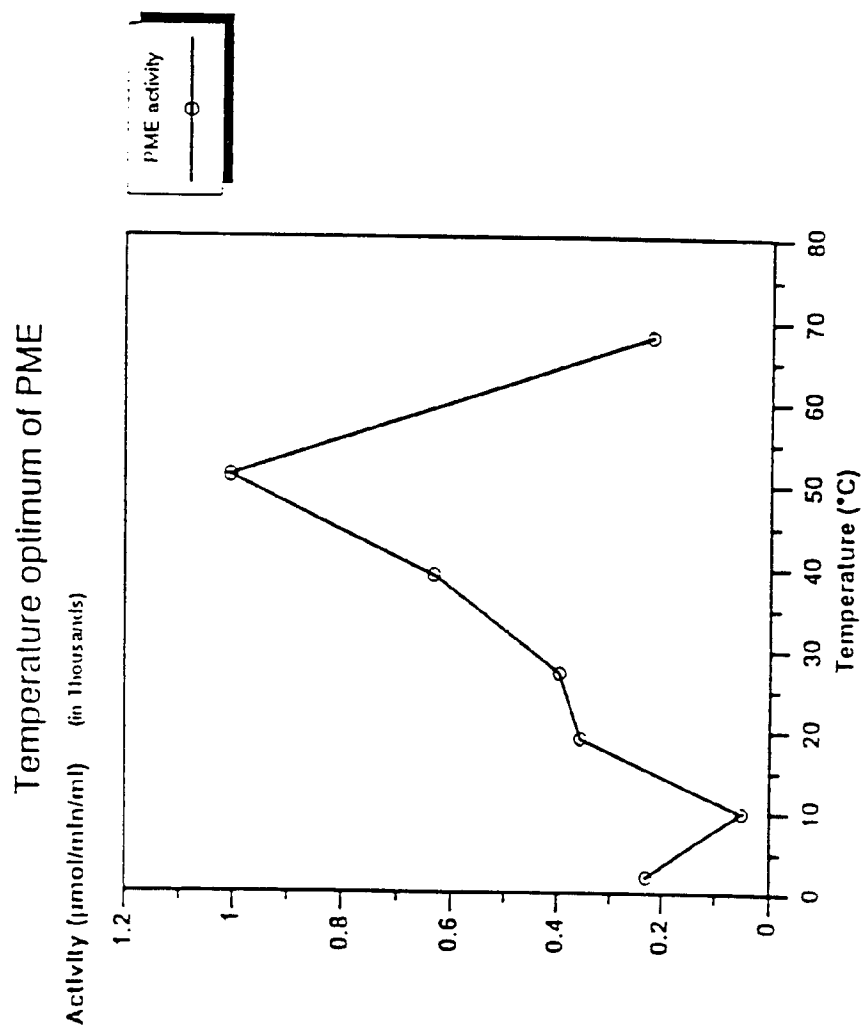
3/18

Fig 3



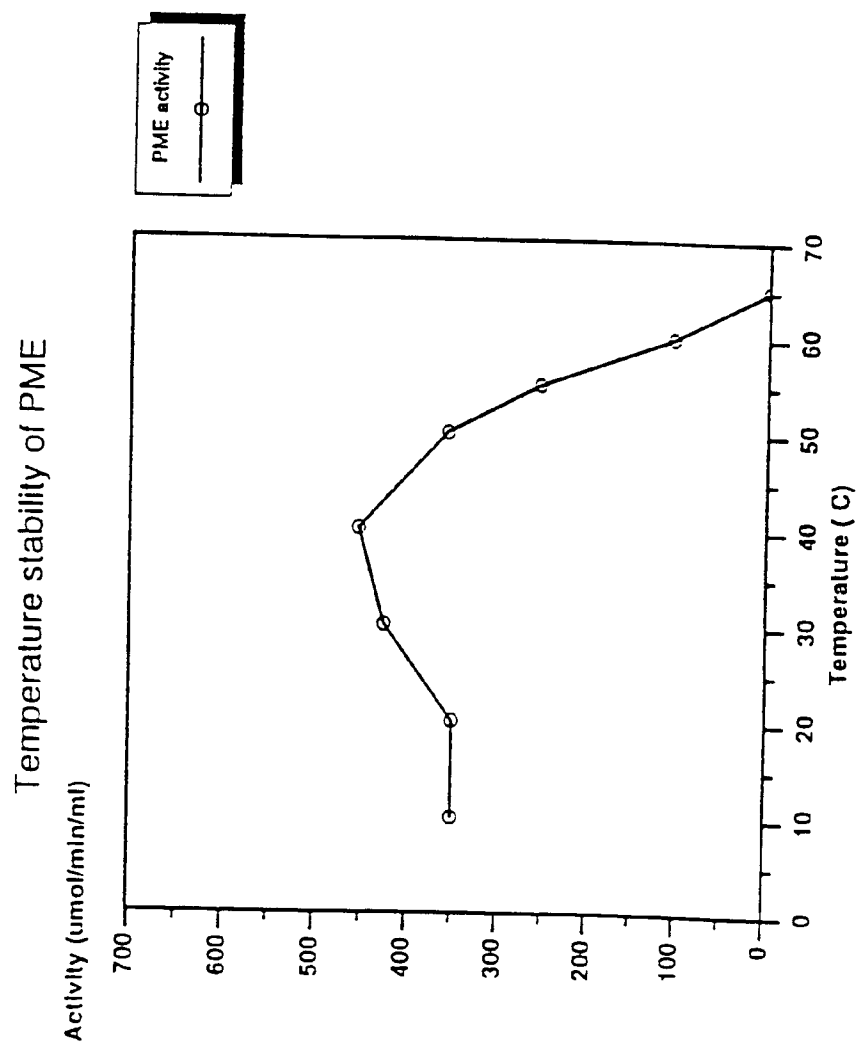
4/18

Fig 4



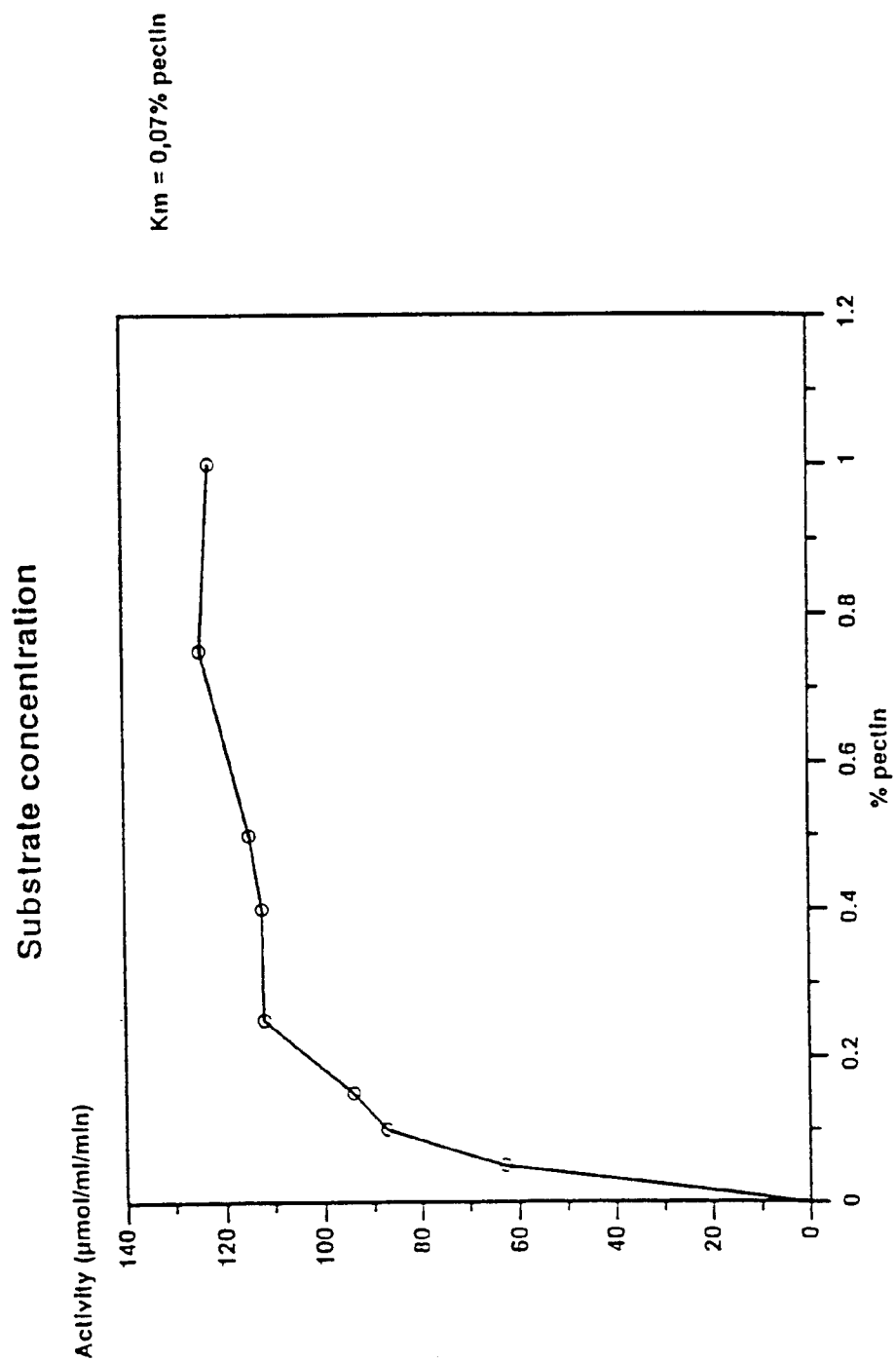
5/18

Fig 5



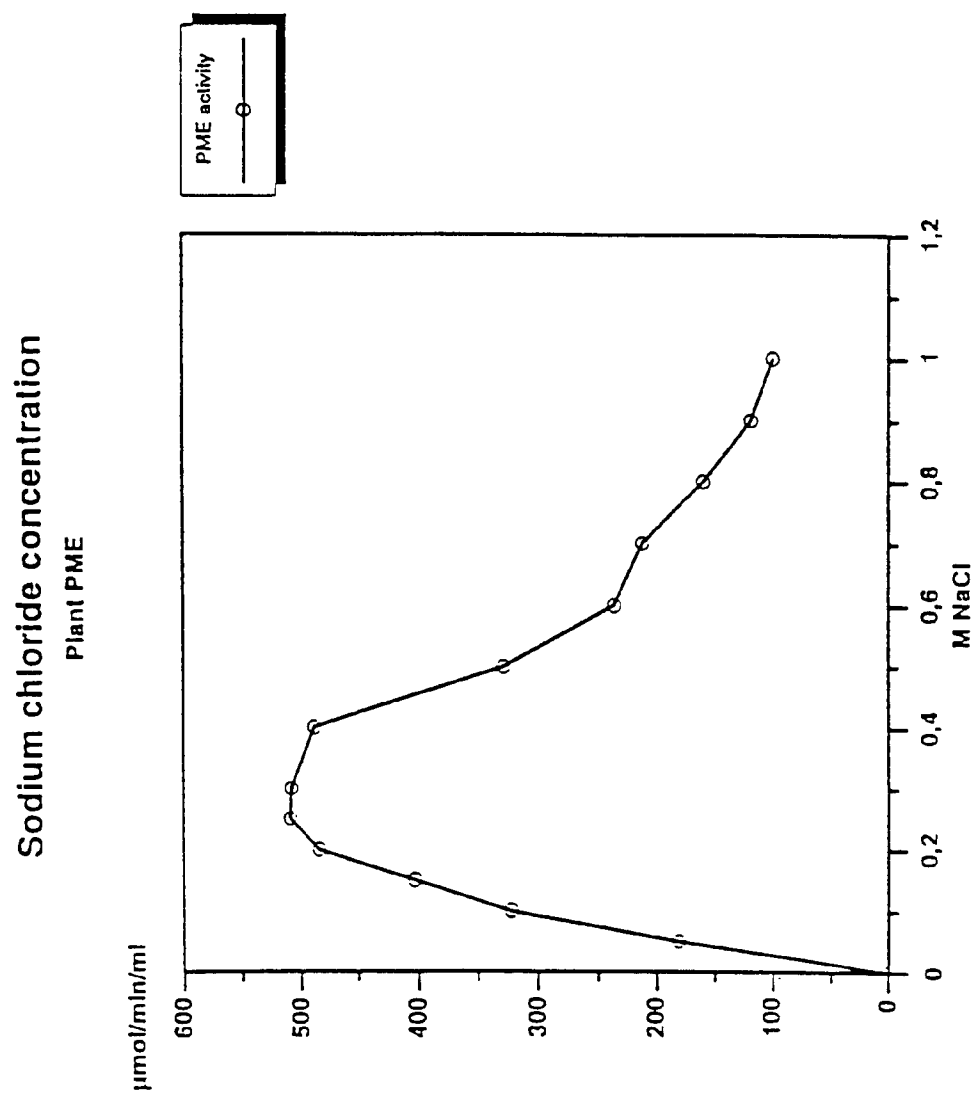
6/18

Fig 6



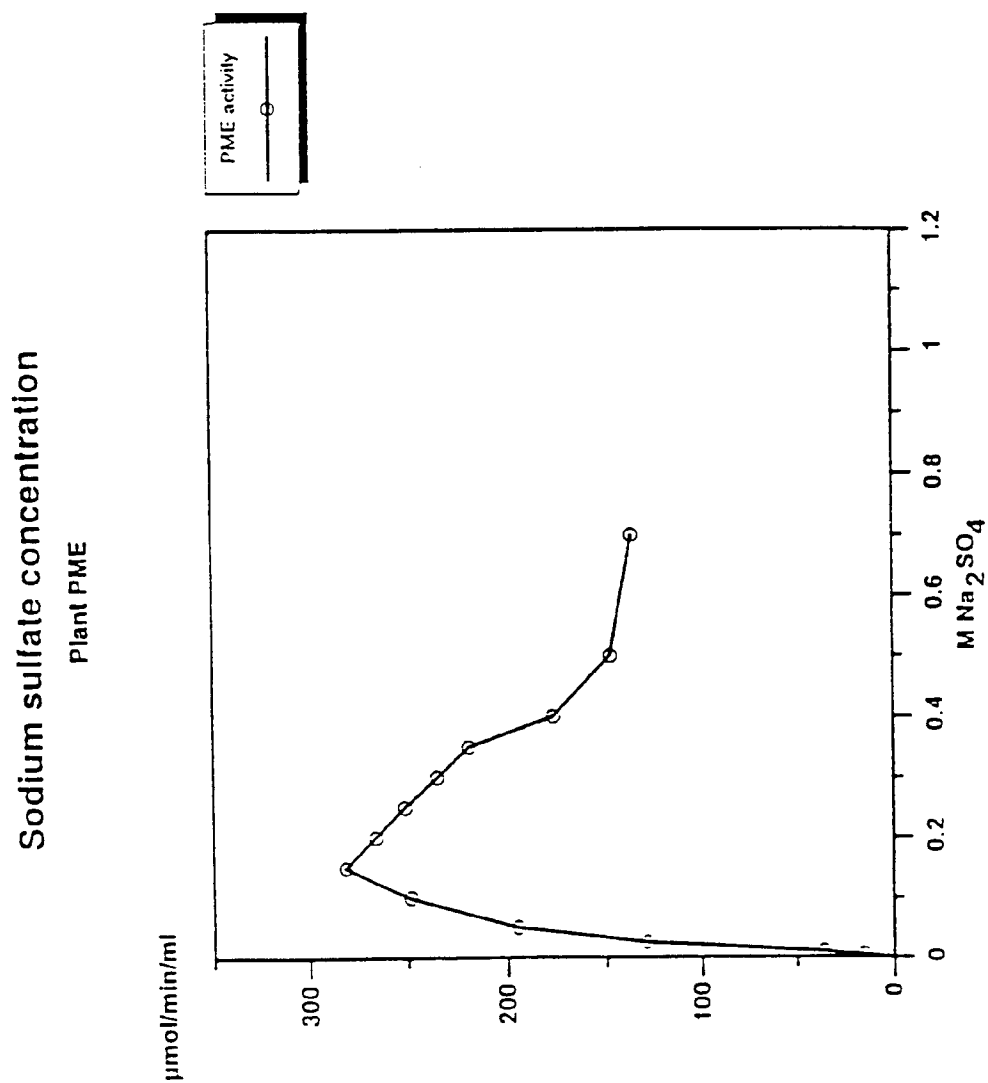
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Fig 7



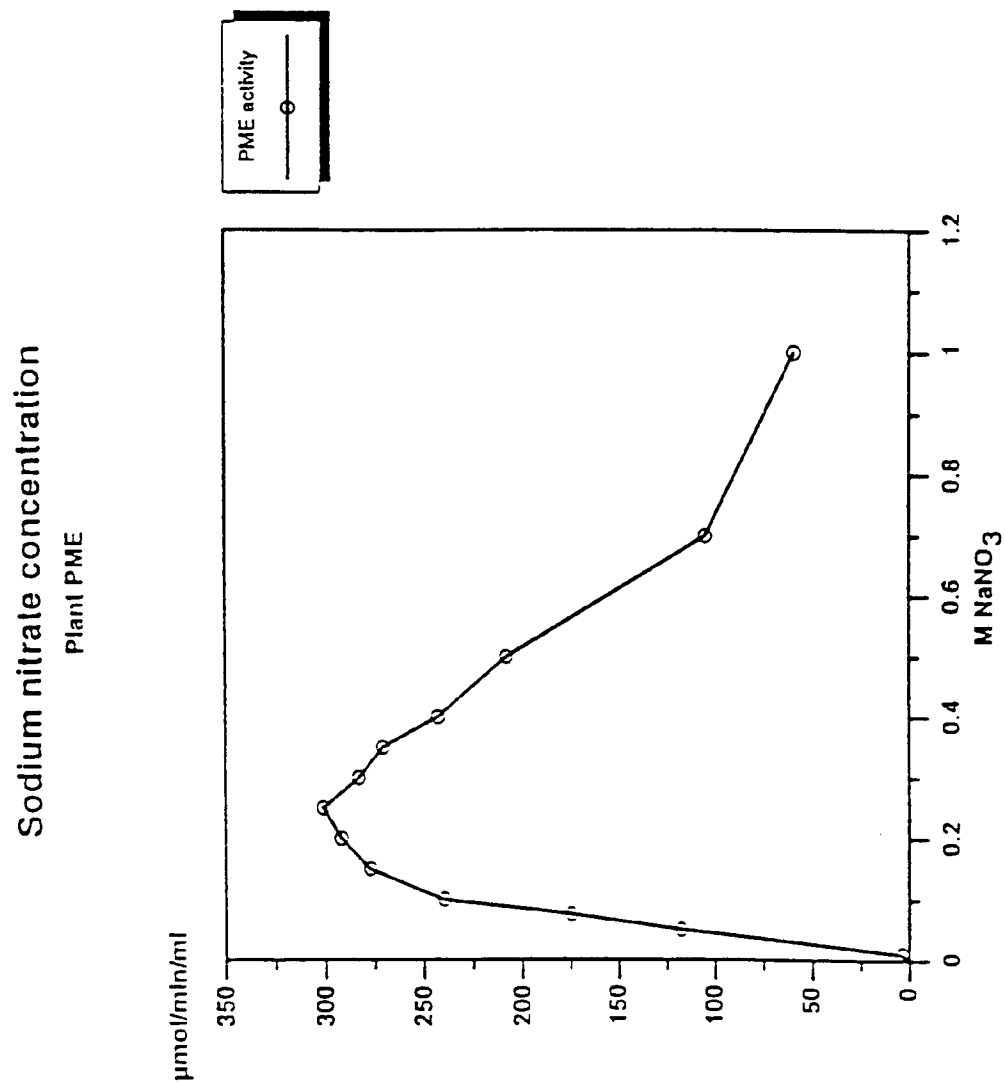
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Fig 8



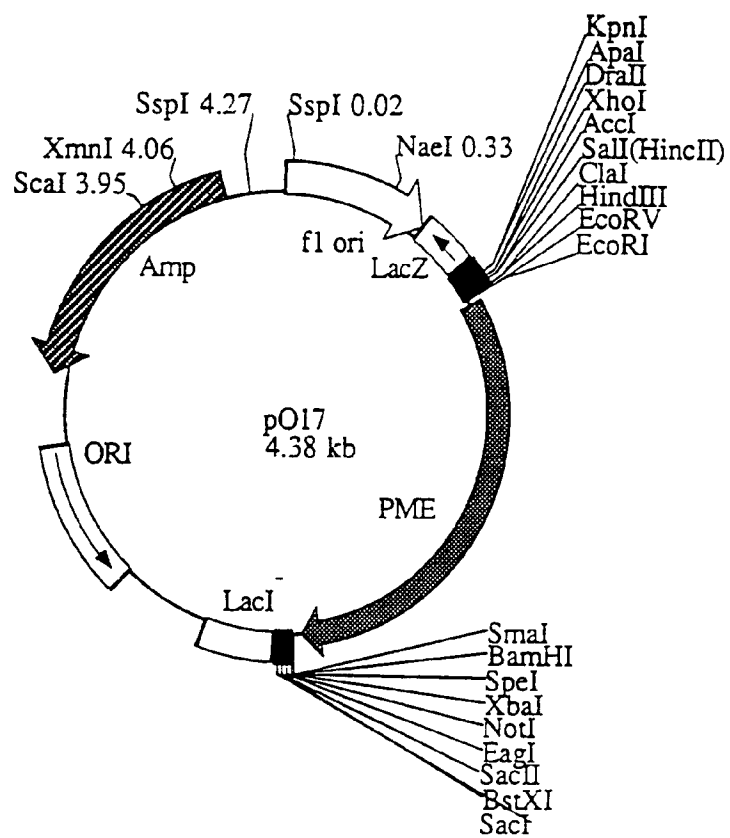
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Fig 9



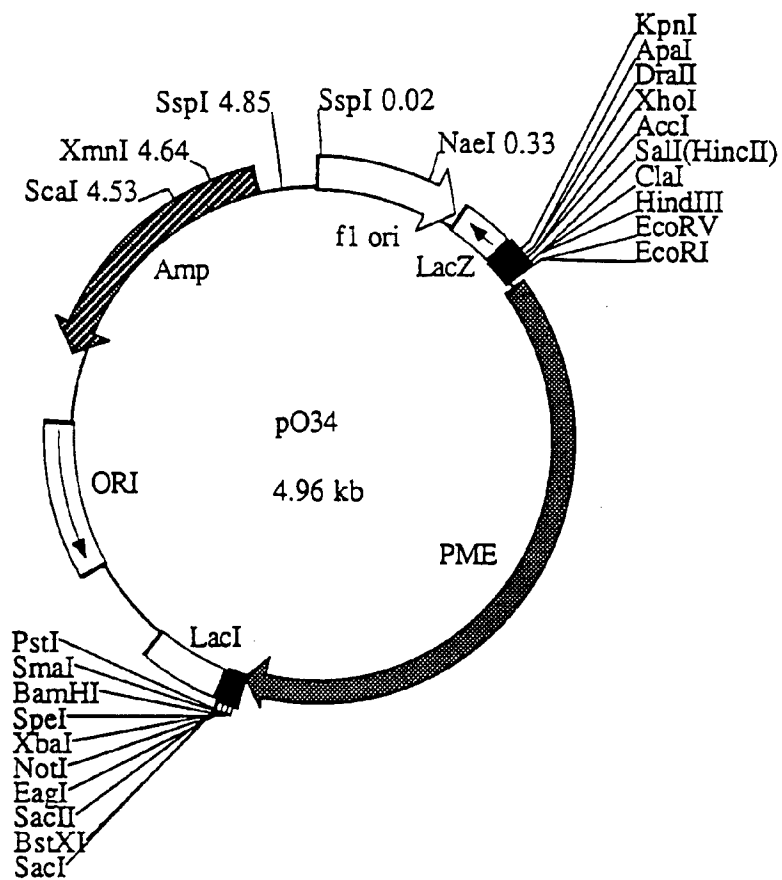
10/18

Fig 10



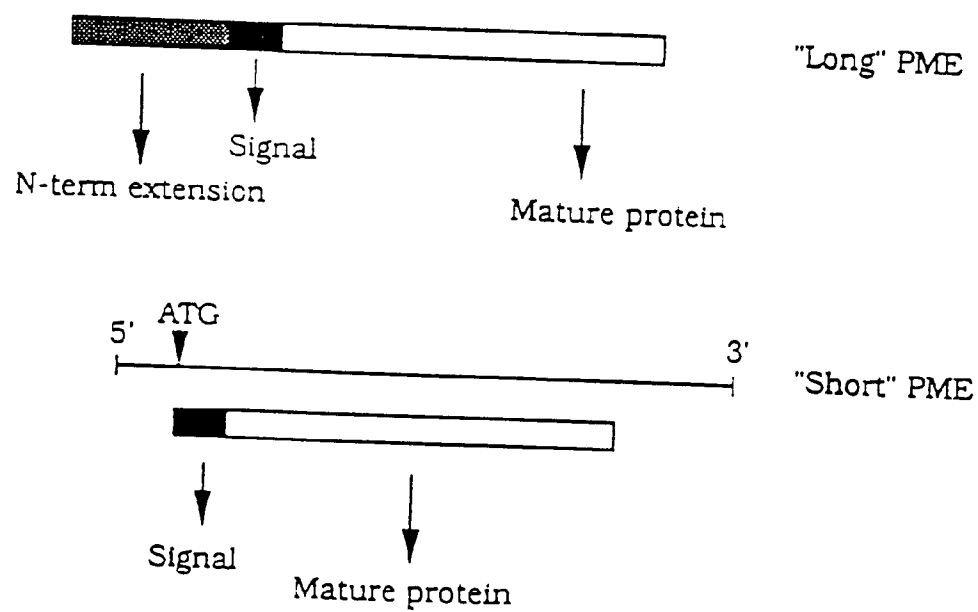
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Fig 11



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Fig 12



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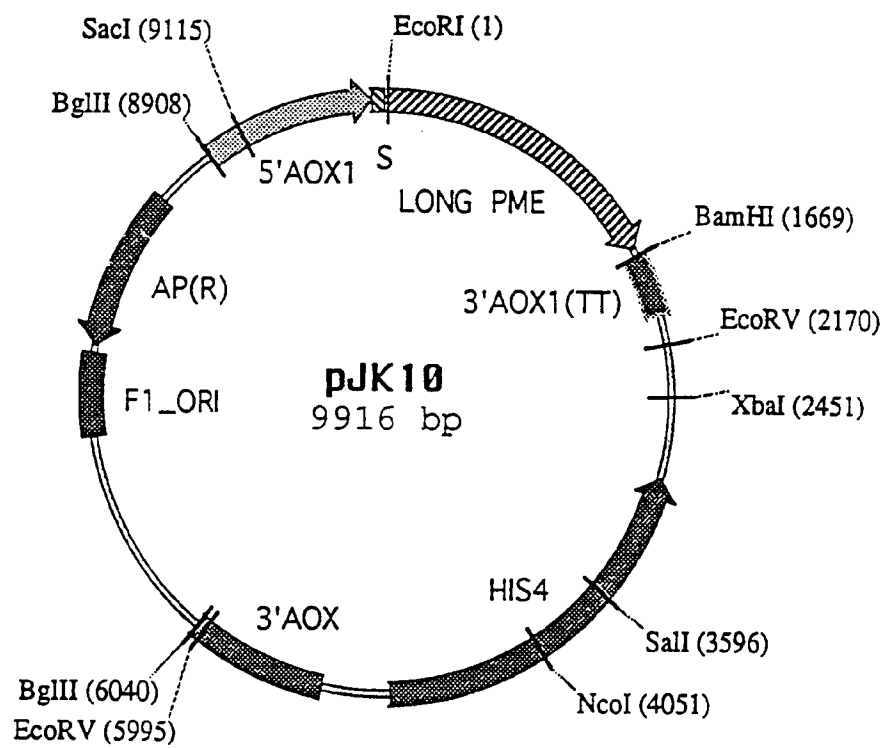


Fig 13

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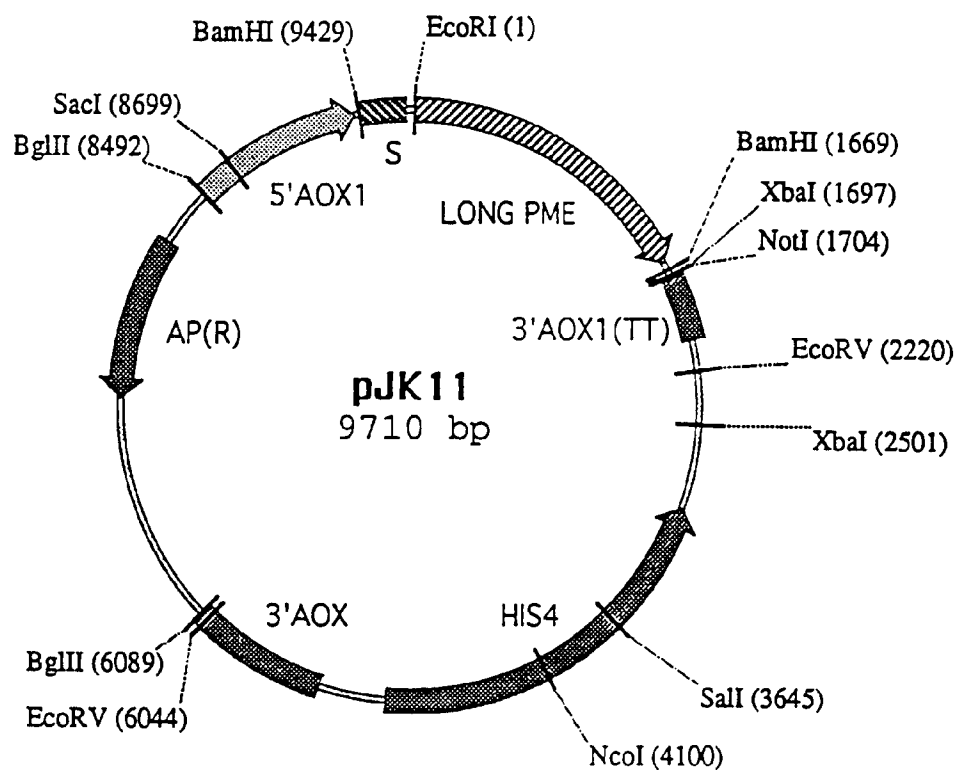


Fig 14

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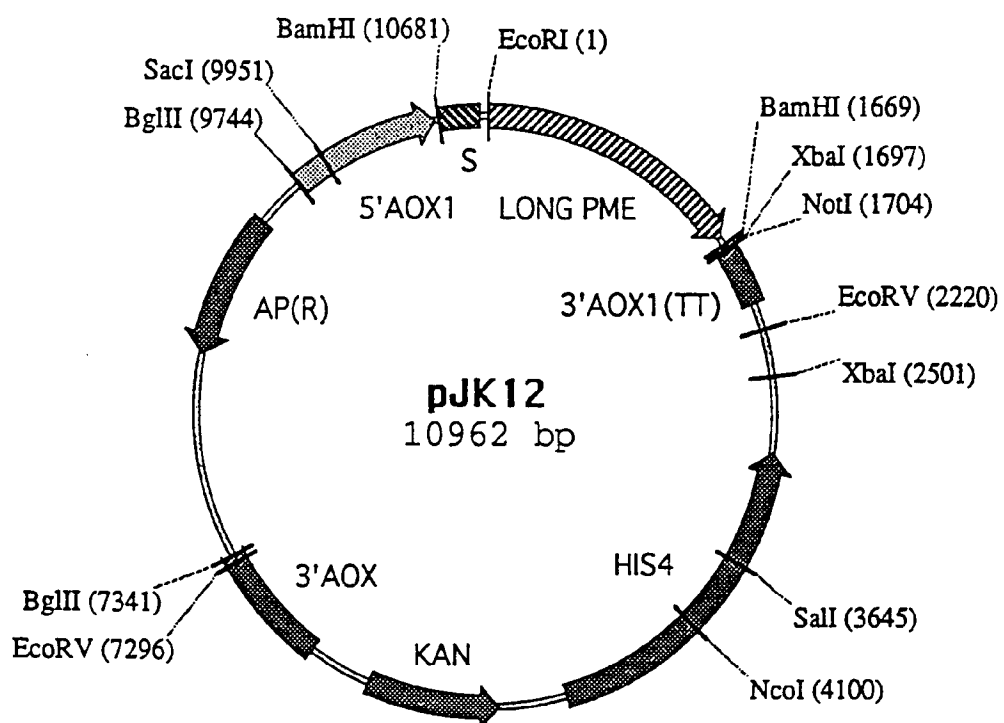


Fig 15

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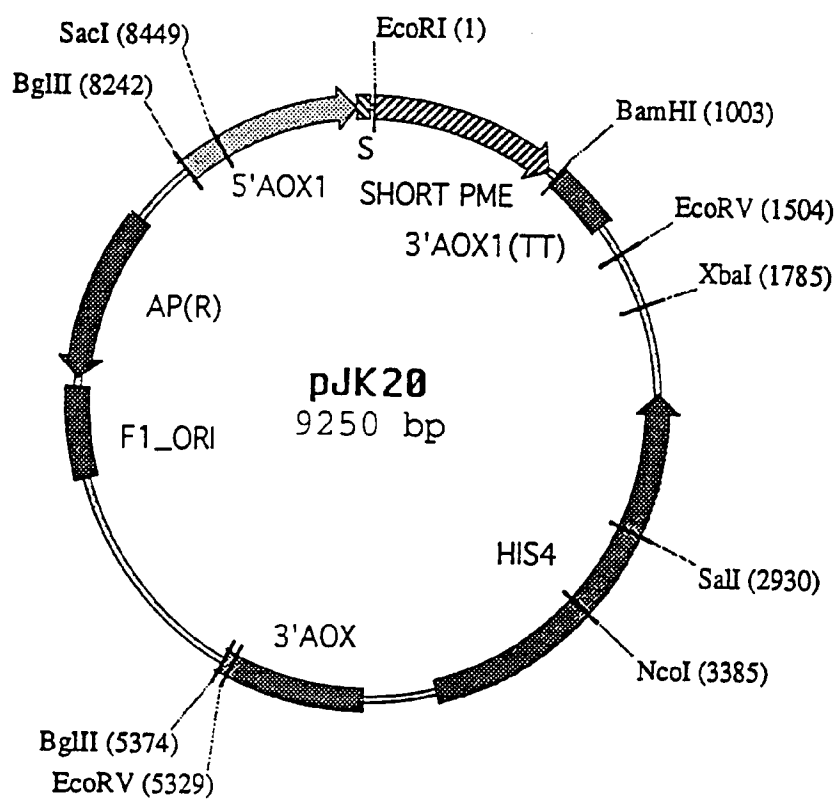


Fig 16

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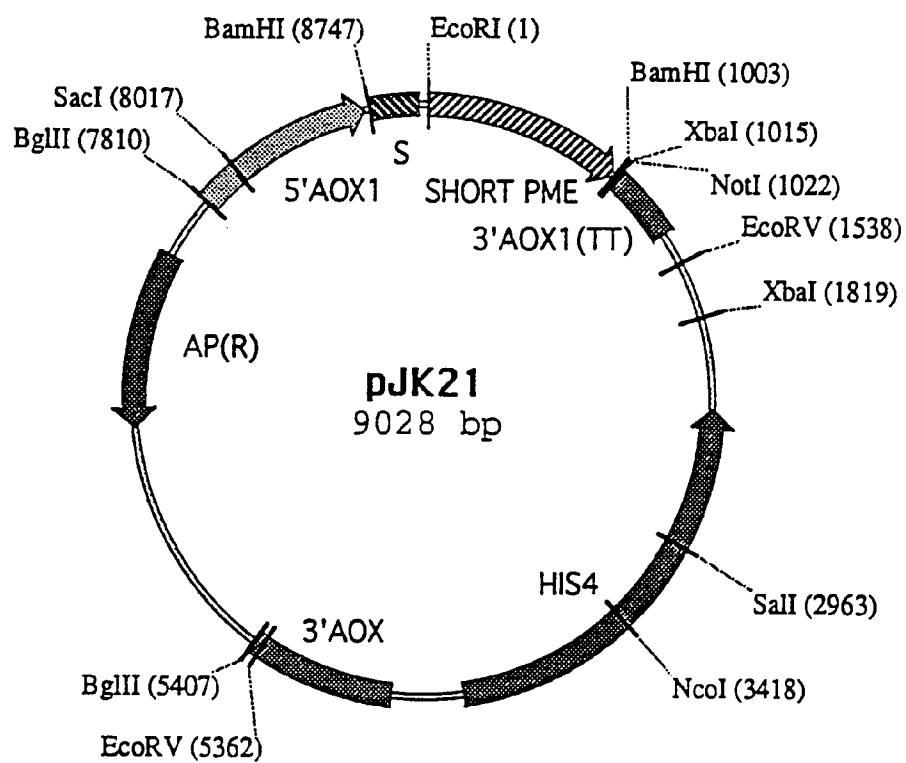


Fig 17

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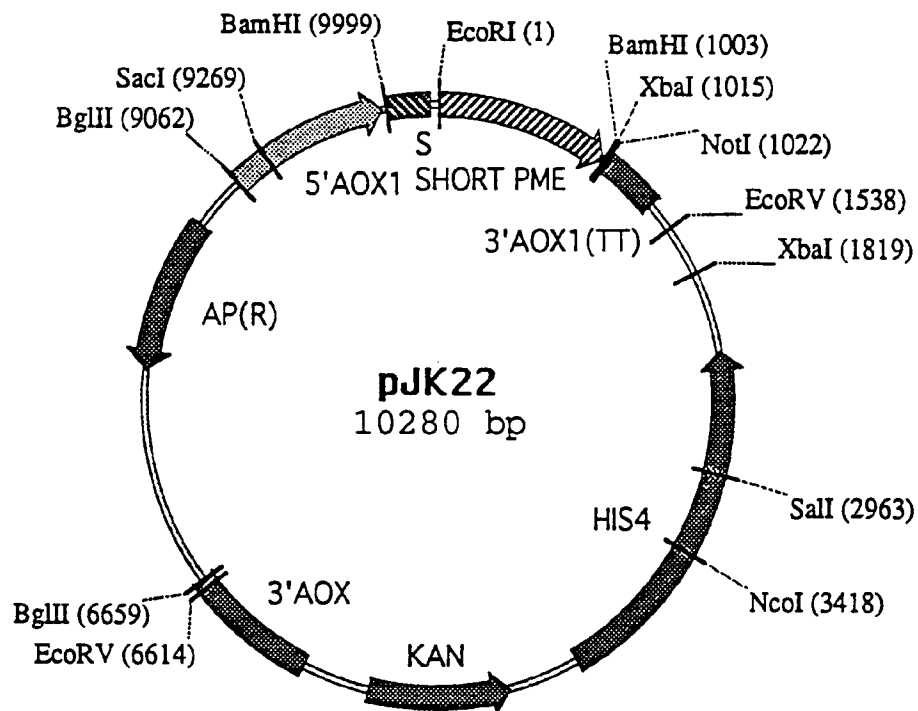


Fig 18

INTERNATIONAL SEARCH REPORT

national Application No

PCT/EP 96/03051

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 A23L1/0524 A23C9/137 A23C9/154 A23C21/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A23L A23C C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	"Food Ingredients Europe - Conference Proceedings" 1994 , PPE , MAARSEN - THE NETHERLANDS XP002016403 Pages 252-256 see the whole document ---	1-21, 40-42,48
X	VOEDINGSMIDDELEN TECHNOLOGIE, vol. 15, no. 20, 1982, ZEIST NL, pages 25-29, XP002016401 A.C.M. VAN HOOYDONK: "De Bereiding van Drinkyoghurt: 2. De Invloed van de Produktsamenstelling" see page 27, left-hand column see table 2 --- -/--	1-21, 40-42,48

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search

18 December 1996

Date of mailing of the international search report

17.01.97

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 Fax (+31-70) 340-3016

Authorized officer

Vuillamy, V

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 96/03051

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	<p>DATABASE WPI Section Ch, Week 9628 Derwent Publications Ltd., London, GB; Class D13, AN 96-271372 XP002016404 & JP 08 112 059 A (NITTA GELATIN KK) , 7 May 1996 see abstract</p> <p>---</p>	<p>1-21, 40-42,48</p>
X,P	<p>EP 0 709 033 A (YAKULT HONSHA KK) 1 May 1996 see claims 1-4 see the whole document</p> <p>---</p>	<p>1-21, 40-42,48</p>
A	<p>FEBS LETTERS, vol. 355, 1994, AMSTERDAM NL, pages 135-139, XP002021517</p>	<p>12,13, 18-34, 36-39</p>
X	<p>see figure 5</p> <p>---</p>	<p>35</p>
A	<p>PLANT MOLECULAR BIOLOGY, vol. 25, DORDRECHT NL, pages 313-318, XP002021518 see the whole document</p> <p>---</p>	<p>12-14, 18-39</p>
A	<p>WO 94 25575 A (NOVO NORDISK) 10 November 1994 see the whole document</p> <p>---</p>	<p>12,18-39</p>
A	<p>DIE NAHRUNG, vol. 19, no. 1, 1985, pages 75-85, XP002016402 R. KOHN: "Die Verteilung der Freien und Veresterten Carboxylgruppen im Pektinmolekül nach Einwirkung von Pektinesterase aus A.Niger und Orangen" see table 1</p> <p>-----</p>	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 96/03051

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please see next page.

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☒ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

- 1) Claims 1-11: Process comprising adding to an acidic environment, which contains at least one protein, a blockwise enzymatically de-esterified pectin, as far as not involving the variants specified in claims 12-48
- 2) Claims 12-48: Process involving a recombinant pectin methyl esterase and means (nucleotide sequence, constructs, vectors...) for producing said recombinant enzyme. Uses of said enzyme.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 96/03051

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0709033	01-05-96	JP-A- 8112058	07-05-96
		AU-A- 3432595	02-05-96

WO-A-9425575	10-11-94	AU-A- 6676794	21-11-94
		EP-A- 0696319	14-02-96
